DOCTORAL THESIS

Uncovering the onshore life of king penguins via energy expenditures: understanding their physiological stress response and the biomechanics of their pedestrian locomotion

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Uncovering the onshore life of king penguins via energy expenditures: understanding their physiological stress response and the biomechanics of their pedestrian locomotion

by
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Abstract

Measuring energy expenditure using respirometery, heart rate and accelerometry can enable hitherto unknown aspects of a species' energetic ecology to be uncovered. Due to the increased use of these methods, rigour is required to improve the accuracy of the results. As they can only feed in the sea, King penguins (*Aptenodytes patagonicus*) need to manage their onshore energetic budget well. During fasting periods, which can last up to one month, heavy individuals need to walk several kilometres to reach their zone of attachment, where they incubate and take care of the egg 24 hours a day. They then need to have sufficient energy reserves to return to sea, swim to the polar front and efficiently fish for prey. Consequently, knowing the energy expenditure of king penguins while onshore is key for understanding their future survival. By investigating the onshore energy expenditure of king penguins, this thesis generates new insights not only into their physiological stress response and the biomechanics of pedestrian locomotion, but also into proxy-based methods of measuring energy expenditure. The cardio-respiratory stress response was defined for this species, with some surprising findings, and the energetic cost of the stress response was demonstrated. Implications for the confounding effect of stressed states on energy proxy calibrations were considered and a standard protocol to alleviate this issue in future studies of king penguin energetics is proposed. The biomechanics and energetics of the pedestrian locomotion were investigated to enhance the understanding of the mechanisms developed to optimise king penguin gait in relation to their body mass. Following investigation of differences in walking between heavy and light penguins, no conclusive explanations were established, though future investigations are suggested to enhance this learning. Finally, using the data collected throughout the thesis, the energy expenditure of early and late breeders was investigated, enabling a better understanding of their energy budgets which can be fed into conservation projects for king penguins.

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Definitions and abbreviations:

§ : Section.

Acclimated: Refers to the physiology and behaviour exhibited with the stressor-induced bias removed.

Acclimation: "The concept that after repeated or chronic exposure to a stressor, an animal no longer considers the stressor to be noxious and reduces its glucocorticoid response." (Romero, 2004)

Activity: Refers to the initial level of movement of the bird when subjected to a stressor, e.g. low for resting, high for walking.

ASTW: Astrid S. T. Willener.

ATP: Adenosine-5'-triphosphate.

DBA: Dynamic body acceleration, inertia of a movement made by accelerometer on an animal, given in one of the tri dimensional axis.

Duty factor: The percent of the total cycle of the stance phase.

Early/late breeders: King penguins arriving at the beginning of the breeding season (after October) are considered early breeders, while birds laying after mid-January are considered late breeders.

FR: Flow rate.

Fullest possible acclimation: Refers to the lowest achieved metabolic rate when measured in the respirometer chamber, calculated as the lowest five-minutes mean $\dot{V}_{O_2}$.

Fullest possible acclimation during daytime: Due to the potential confound of circadian rhythms affecting metabolism, the lowest achieved five-minutes mean $\dot{V}_{O_2}$ during daytime while measured in the respirometer chamber was calculated.

GCOT: Gross cost of transport, described as the relationship between metabolic rate and speed of walking, i.e. $y = a\,x + b$, where x is the speed (m/s) and $\boldsymbol{y}$ is the energy expenditure (J/s).

NCOT: Net cost of transport (J/m), energy used to move a unit distance. Slope of the GCOT function.

O_2: Oxygen.

CO_2: Carbon dioxide.

Level of acclimation achieved by previous studies: Defined as the first five-minute of a stable period of 20-minutes of $\dot{V}_{O_2}$ obtained one hour after the occurrence of the stressor.

Level of acclimation achieved by using the protocol found in this study: Refers to the lowest $\boldsymbol{\dot{V}_{O_2}}$ obtained within 90 minutes.

Motion: Refers only to movement due to the behavioural response ('fight or flight', Cannon, 1929; or the updated 'freeze, flight, fight or fright' response, Bracha et al., 2004). Motion is the difference in movement between the unstressed and stressed states.

Movement: Is used in this thesis as a general term for any contractions of the striated muscle, including physical 'activities' or 'motions'.

ODBA: Overall Dynamic Body Acceleration.

Overall stress response: Refers to any physiological and behavioural changes due to the presence of a stressor (including change in motion).

PCOT: Postural cost of transport. Energy expended to maintain the posture of locomotion, represented as the extrapolated intercept *b* of NCOT minus resting metabolic rate.

PCr: Phosophocreatine.

SBA: Static body acceleration, estimated from recordings of an accelerometer logger instrumented on an animal, which can provide information on the posture of the animal.

Shift: During the breeding season, one member of a king penguin pair takes care of the egg or chick onshore, while its partner is fishing at sea. When the sated partner returns, an exchange of the egg or chick occurs. This exchange is called a shift. For instance, the first shift concerns the exchange of the egg layed by the female to the male. An average of 10 shifts occurs during the breeding season (5 while incubating an egg and 5 while brooding a chick).

Stance phase: The phase during which the foot is on the ground.

Step: Defined as the time between toe-off and the next initial contact the same foot makes with the ground.

Stress response: "The physiological, hormonal and behavioural changes that enable an animal to cope with a stressor''(Romero, 2004). However this thesis only looked at the cardio-respiratory and behavioural (i.e. in term of movements) stress responses.

Stress response per se: Refers to the physiological and behavioural stress responses which are a direct result of the stressor. This response does not include the physiological response due to increased body motion associated with the stressor.

Stressed: Refers to the physiology and behaviour exhibited under presence of an anthropological stressor.

Stressor: "A noxious or unpredictable stimulus that causes a stress response" (Romero, 2004).

Stride: A cycle of stride is defined by the temporal interval of two successive similar positions of the same foot, e.g. from the initial contact to the next one. One stride contains two steps.

Swing phase: The phase during which the foot is off the ground.

Unstressed: Refers to the physiology and behaviour exhibited without the presence of an anthropological stressor.

VAV: Vincent A. Viblanc.

VeDBA: Vectorial Dynamic Body Acceleration.

$\dot{V}_{O_2}$**:** Rate of oxygen consumption (ml/min).

1. General Introduction

1.1 What is Energy?

To quote Albert Einstein: "Energy cannot be created or destroyed, it can only be changed from one form to another." In biology, energy is the currency of life (as pointed out by Dawkins, 1976), defined as "the ability to maintain or increase order in a system" by Hill et al. (2008). Without it, there is no anabolism or catabolism, i.e. no metabolism necessary for maintaining life. Organisms need to acquire, transform and use energy for all biological processes (Brown et al., 2004). If acquisition is higher than expenditure, energy is typically stored, predominantly as fat, for later use. If expenditure is higher than acquisition, the animal is starving, leading to its death if this net energy loss is maintained for too long. Energy expenditure thus reflects the evolutionary fitness of organisms in maintaining an optimal trade-off between energy intake and output (Goldstein, 1988, Tolkamp et al., 2002), especially for species with restricted areas or episodic resource availability. In recent years, measurements of the energy expenditure of animals have been used with increasing frequency, particularly in ecological, biomechanical and conservation contexts (Shepard et al., 2008, Halsey et al., 2008d, Arnould et al., 1996, Halsey, 2011, Maloiy et al., 1986), as they enable a better understanding of life history (Hall et al., 2001), trophic flow (Lowe, 2002), biogeography (McNab, 2002) and behavioural strategies (Hinch and Rand, 1998) as mentioned by Gleiss et al. (2010).

1.2 How can energy expenditure be measured and estimated?

The natural currency of life is represented as adenosine-5'-triphosphate (ATP). ATP is a reactive molecule whose chemical bindings are energetically full (Equation 1-1), and is used to process metabolic endothermic reactions. Once the reaction has been completed, ATP is converted into its precursor, via metabolic pathways, and it is thus recycled for further use. In animals, the predominant metabolic pathway that generates energy is aerobic oxidative respiration (Hill et al., 2008).

Equation 1-1

$$ATP \rightarrow ADP + P_i + 7.3 \text{ kcal/mol ATP (Biewener, 2003)}$$

1.2.1 Aerobic respiration and ATP formation

Aerobic respiration consists of a series of chemical reactions that occur within the cell, especially in the mitochondria. The mitochondria are sometimes described as the energy factories of an organism, as they transform oxygen (O_2) and carbohydrates (e.g. polysaccharides) into ATP, carbon dioxide (CO_2) and water (Hill et al., 2008) (Equation 1-2 and Figure 1.1). Different sources of ATP are discussed in the chapter two 'General Methods' (in §1.2.2 and § 2.3.1.1).

Equation 1-2

$$C_6H_{12}O_6 + 6\ O_2 + 38P_i + 38ADP \rightarrow 6\ CO_2 + 6\ H_2O + 38\ ATP,$$

$$\Delta G° = -420 \text{ kcal (as heat) (Eckert, 1988)}$$

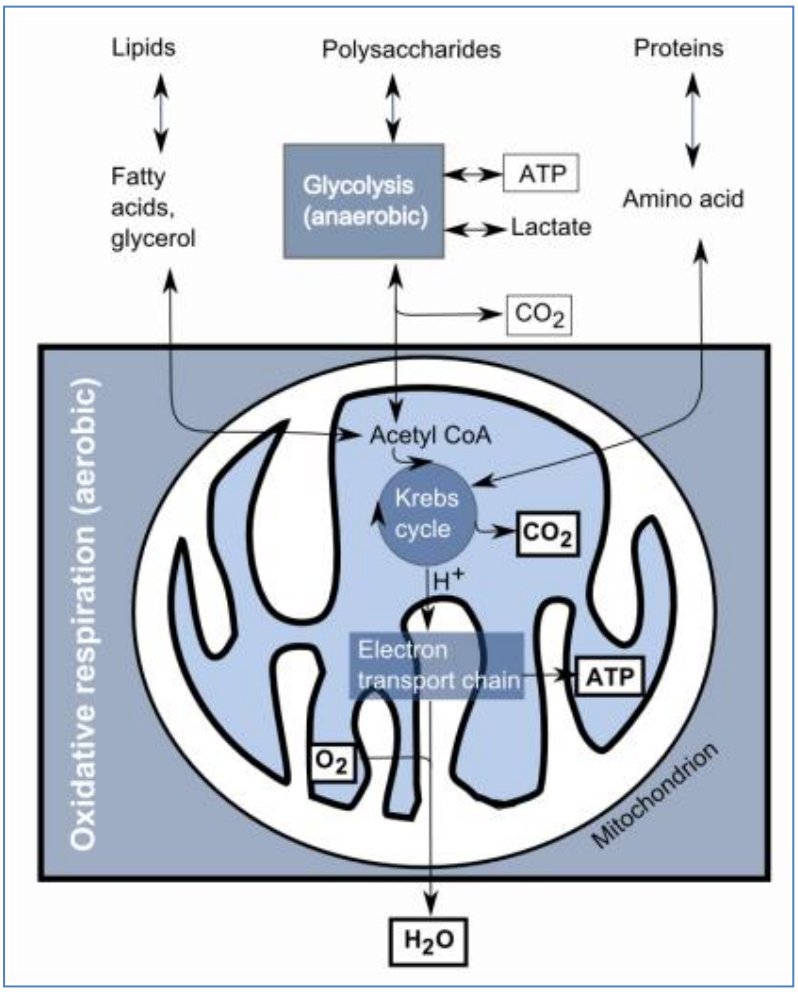


Figure 1.1 ATP formation from degradation of lipids, polysaccharides and proteins, highlighting oxidative respiration (modified from Eckert, 1988).

The equation for aerobic respiration (Equation 1-2) shows that ATP production (i.e. the quantity of energy required) is directly proportional to heat production or gas intake and output. Direct calorimetry measures the heat production of an organism placed in an adiabatic chamber and was the first technique used to measure energy expenditure (Lavoisier, 1777, Lavoisier and Laplace, 1780, Lavoisier and Seguin, 1789). However, even with modern technology this seemingly simple measurement is not easily recorded with accuracy. The common alternative method is to measure exchanges in the respiratory gases (O_2/CO_2 + H_20, Equation 1-2). This method of indirect calorimetry (O_2 consumption or CO_2 production can subsequently be converted into estimates of power output using assumptions of the metabolic substrate) is typically called respirometry and is almost always conducted within an experimental environment. Respirometry is one of the most accurate measurements of energy expenditure when the study organism is respiring predominantly aerobically. Several other methods have been developed to estimate respiratory gaseous exchanges, each having their pros and cons. The scale of measurement (seconds, days) depends on the technique, some techniques are invasive, and some require expensive material or surgical knowledge, etc. Some of the most commonly used methods are doubly labelled water, body mass loss (Portugal and Guillemette, 2011), heart rate (Green, 2011), accelerometry (Halsey et al., 2011) and respirometry (Lighton and Halsey, 2011). For the work in this thesis, which centres on short-term measurements of energy expenditure, respirometry, the heart rate technique and the accelerometry technique were employed.

1.2.2 Respirometry

The proportions of O_2 and CO_2 breathed in and out from the subject animal are measured. This measurement can then be converted into the quantity of carbohydrates burnt and thus the amount of ATP can be extrapolated. The respiratory gas exchange of the subject animal is captured and analysed, by way of the animal either breathing into a mask or into a respirometry chamber within which it has been placed. A tubing circuit connects the expired air to the respirometer, which measures the proportions of O_2 and CO_2 entering and leaving

the box. Exhaled air results in a decrease in O_2 concentration and an increase in CO_2 concentration in the sample air. Different metabolic fuels result in different ATP yields however calculating the respiratory exchange ratio, RER ($\frac{Rate\ of\ CO_2\ produced}{Rate\ of\ O_2\ consumption} = \frac{\dot{V}_{CO_2}}{\dot{V}_{O_2}}$) enables an estimation to be made of the substrate oxidized (Dejours, 1981; e.g. 0.7–1.0 for aerobic catabolism of fats and carbohydrates, respectively and proteins have intermediate values based on their mix of amino acids. Lighton, 2008). An RER lower than 1 indicates that the exercise is performed aerobically, indicating that the use of respirometry is appropriate to estimate energy expenditure. A requirement of the respirometry technique is that the animal must be subjected to an experimental material or environment. As each behaviour requires different energy expenditure, the specific studied behaviour should be reproduced by the animal in the laboratory environment. For instance to measure the walking energy expenditure, the experiment should involve a treadmill to enable the animal to walk while the respiratory gas exchange is measured. Ideally, the exhibited behaviour should be done in a similar manner as the natural behaviour. Additionally, changes in energy expenditure may be due to factors such as thermoregulation state (Kamau and Maloy, 1982), for this reason Green (2001) suggested that the natural condition should be mimicked as far as possible in the laboratory. To estimate the daily energy budget of an animal, the daily time budget of the animal needs to be done and then converted into energy expenditure via the calibration of energy expenditure collected in the laboratory. However, to create a time budget, the animal needs to be observed in the wild and with the least possible disturbances, which is not always technically possible. Thus, for this reason, proxies that estimate enable energy expenditure are used. These proxies are typically the heart rate (Figure 1.2) method and the increasingly used accelerometery (Figure 1.3) method, which enable measurement of the animal outside the experimental environment, without the need to follow the animal.

1.2.2.1 Limitations

However, the method has some limitations that need to be taken into account. For instance, energy expenditure is not constant for each behaviour. Factors such as sex (Green et al., 2001), thermoregulation state (Kamau and Maloy, 1982), physiological state (e.g. reproductive state) and nutritional state (Froget et al., 2001, Green et al., 2001) influence the entire organism physiology and thus the energy expenditure. External factors also influence energy expenditure, e.g. the walking surface (Pinnington and Dawson, 2001). Consequently, mimicking the parameters of an animal's natural condition when measuring energy expenditure logically improves the energy expenditure estimation. Another option would be to use several bivariate calibrations to estimate energy expenditure of a species.

1.2.3 Heart rate

Blood is the medium by which O_2 is transported to the cells that require it. As Fick's convection law explains (Equation 1-3), rate of oxygen consumption ($\dot{V}_{O_2}$) transferred from the arterial blood to the cells is proportional to the product of the difference in O_2 concentration between the arterial and venous blood, and the cardiac blood flow, the latter being itself the product of heart stroke volume and heart rate (Fick, 1870). If the difference of O_2 concentration between the arterial and the venous blood and stroke volume are constant, heart rate varies linearly with $\dot{V}_{O_2}$. Experimentally, the relationship between $\dot{V}_{O_2}$ and heart rate is collinear in many species (see Green, 2011 for a definitive list until 2011), enabling an estimation of $\dot{V}_{O_2}$ from heart rate data collected in a free-ranging animal, using a calibration relationship obtained in the laboratory. Such calibration involves simultaneously measuring $\dot{V}_{O_2}$ and heart rate of the subject animal during activity at different levels (typically induced by a treadmill or water flume), thus in captive conditions. Consequently, calibration equations are described by the bivariate relationship between $\dot{V}_{O_2}$ and heart rate.

Equation 1-3

$$\dot{V}_{O_2} = HR * V_s * (C_{aO_2} - C_{vO_2})$$

;where $\dot{V}_{O_2}$ is rate of oxygen consumption, HR is heart rate, V_s is stroke volume, C_{aO_2} is oxygen content of arterial blood , and C_{vO_2} is oxygen content of mixed venous blood.

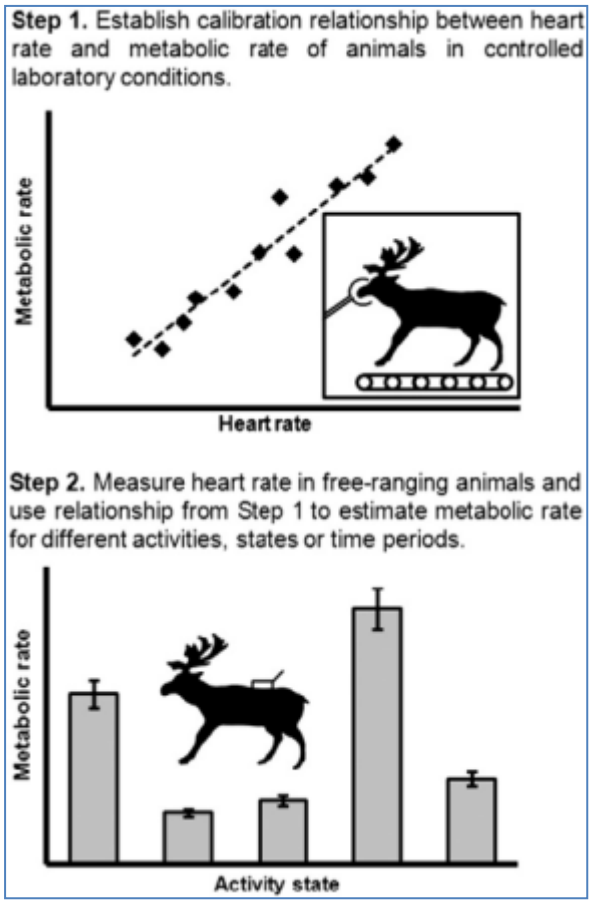


Figure 1.2 Application of the heart rate technique. "Schematic diagram showing the two steps necessary for the full application of the heart rate method used to estimate metabolic rate in free-ranging animals. The ideal or "gold standard" approach would be to use the same individual in both steps. However a common approach is to use a different group of animals in each step, which necessitates the creation of a group relationship in Step 1, to be used in Step 2" (Green, 2011). See text for further details.

1.2.3.1 Limitations

Some of the factors modifying energy expenditure affect the $\dot{V}_{O_2}$- heart rate calibrations (see Green, 2011 for further details) such as sex (Green et al., 2001), thermoregulation state (Kamau and Maloy, 1982), physiological state (e.g. reproductive state) and nutritional state (Froget et al., 2001, Green et al., 2001). Indeed, for instance, thermoregulation (due to a change in activity level, Butler et al., 2000, Froget et al., 2002, or due to the environmental temperature, Green, 2011) affects the stroke volume (see Equation 1-3) via the sympathetic/parasympathetic systems to keep the body in homeostasis. Other individual

factors such as body condition achieved by training (e.g. wild versus captive individuals, Green, 2011) also influence stroke volume and thus the $\dot{V}_{O_2}$- heart rate calibrations. Consequently, it is advisable to mimic the parameters of an animal's natural condition (e.g. temperature) as closely as possible in the laboratory during calibration experiments (Green, 2011), as well as using the same individual while defining the calibration relationship and when collecting data while they are free-ranging. However, if the same animal cannot be used for both conditions, it is strongly advised to use a calibration relationship as well as free ranging data of a group of animals, which will avoid problems associated with differences between individuals (Green, 2011)

1.2.4 Accelerometry

Another method for estimating energy expenditure, which is becoming more widely used, is measuring levels of body movement in a subject animal via recordings of triaxial acceleration from an instrumented accelerometer. As explained in Gleiss et al (2010), energy is defined in physics by being the potential to do work. While in biology, energy is stored in the form of chemical bonds of ATP (§1.2.1) and used, for instance, to execute movements. Body movements (mechanical work, W) are created by muscle contractions, which are a mechanism of shortening muscular filaments, themselves being activated by the reduction of ATP into ADP + P_i,i.e. chemical energy. The rate at which this mechanical work is done (and thus the rate of energy used) is termed the mechanical power (P). Consequently, highly active behaviour requires more ATP than low activity behaviour. Several studies have demonstrated correlations between $\dot{V}_{O_2}$ and body movement (i.e. described as accelerations in physics). Although movement (i.e. locomotion) has been shown to be a highly variable component of animal time budgets (Garland Jr., 1983), it is still a highly important component in animal energetic budgets (e.g. Birt-Friesen et al., 1989, Tatner and Bryant, 1986). However, the accelerometry technique is not useful for comparing or estimating energy expenditure for sedentary behaviours (see § 1.3 for further information about the

limit of $\dot{V}_{O_2}$-acceleration calibration). The physical link between acceleration produced by muscular contraction and mechanical power (and thus energy expenditure) is presented in Newton's laws (Equation 1-4 and Equation 1-5). Acceleration is the difference between velocities (Δv) per time (t) (Equation 1-4), and the mechanical equivalent of energy expenditure is power (P), which is the work (W) made per unit time (in Watt/s) (Equation 1-5).

Equation 1-4

$$\vec{a} = \frac{\Delta v}{t} = \frac{\frac{d}{t}}{t} = \frac{d}{t^2}$$

Additionally, work equals force ($\boldsymbol{F}$) multiplied by distance ($\boldsymbol{d}$) to move an object (in Nm or J). A force is described as mass ($\boldsymbol{m}$, in kilogram) multiplied by its acceleration ($\boldsymbol{a}$) and velocity is distance (in metres) covered per unit time ($\boldsymbol{t}$ in seconds). However, velocity can also be explained relative to acceleration. From Equation 1-4, proceeding to the integration of velocity by time results in an equation defining the rectlinear movement uniformly accelerated.

Equation 1-5

$$P = \frac{W}{t} = \frac{\vec{F} * \vec{d}}{t} = \frac{(m * \vec{a}) * d}{t} = m * \vec{a} * \vec{v} = m * \vec{a} * (\vec{a}t + \vec{v}_0)$$

Therefore, any mechanical work performed is the proportional result of the acceleration (their magnitude and duration) assuming that the mass of the object does not change. The power is also influenced by the velocity of the centre of mass at time 0 ($\vec{v}_0$), however locomotion gaits showing a 'low dynamic' activity level (Gleiss et al., 2010), as in human walking, has shown that the assumption of $\vec{v}_0$= 0 results in a good estimate (Meichtry et al., 2007). Gaits such as galloping, as in horses (*Equus caballus*), for example, require an estimation of $\vec{v}_0$ (Pfau et al., 2005).

A common method for biologists to express the level of body movement of a motile animal is a single measure which integrates the acceleration measured in all available spatial dimensions. Two different ways to measure levels of body movement exist: summing the absolute value of the dynamic body acceleration of the three axes (Further details in chapter two 'General Methods') or calculating the norm of the resulting vector from the three dynamic partial accelerations. The first method leads to 'overall dynamic body acceleration' (ODBA), which has already been widely used (e.g. Halsey et al., 2009b, Fahlman et al., 2008, Wilson et al., 2006; Figure 1.3), while the second is called the 'vectorial dynamic body acceleration' (VeDBA) (Qasem et al., 2012). See Equation 1-6 and Equation 1-7.

Equation 1-6

$$ODBA\,[g] = |DBA_x| + |DBA_y| + |DBA_z|$$

Equation 1-7

$$VeDBA\,[g] = ||\mathrm{DBA}|| = \sqrt{{DBA_x}^2 + {DBA_y}^2 + {DBA_z}^2}$$

; where DBA is dynamic body acceleration (i.e. inertia of a movement in one of the tri dimensional axis) and the subscripts represent each of the three axes (further details about calculation of DBA are discussed in the chapter two 'General Method'). Several studies have already shown a good correlation between $\dot{V}_{O_2}$ and ODBA (Figure 1.3)

1.2.4.1 Limitations

Accelerometry also has its limitations. Any changes in energy expenditure independent of activity will lead to biased estimations, when the relation $\dot{V}_{O_2}$- accelerometer is used. Energy expenditure may be underestimated due to post-absorptive state, if the animal is growing, gestating, outside of its thermal neutral zone, or if carrying an infant or another load. When the animal is soaring or on moving water, energy expenditure may be overestimated (Halsey

et al., 2011). Furthermore, changes in gait and mechano-chemical efficiency (defined by Alexander and Goldspink, 1977) have been shown to modify the relationship between $\dot{V}_{O_2}$ and acceleration (Gleiss et al., 2010). Indeed, as shown by Newton's laws, a change in mass influences mechanical power. Furthermore not all mechanical power includes movement. Indeed, isometric contractions generate force but not movement, whereby ATP is used without mechanical power, i.e. acceleration (Gleiss et al., 2010, Alexander and Goldspink, 1977). Additionally, different muscle fibre types may have different efficiencies, which lead to a variation in energy consumption depending on which filaments are used (Gleiss et al., 2010, Rome and Sosnicki, 1990). This may explain why changes in gait do not follow a linear $\dot{V}_{O_2}$- ODBA calibration as seen in humans (Halsey et al., 2008d). Also, the effects of wind or changes in topography such as incline on accelerometric data have not yet been widely tested (e.g. Halsey et al., 2008d, Terrier et al., 2001, Campbell et al., 2002).

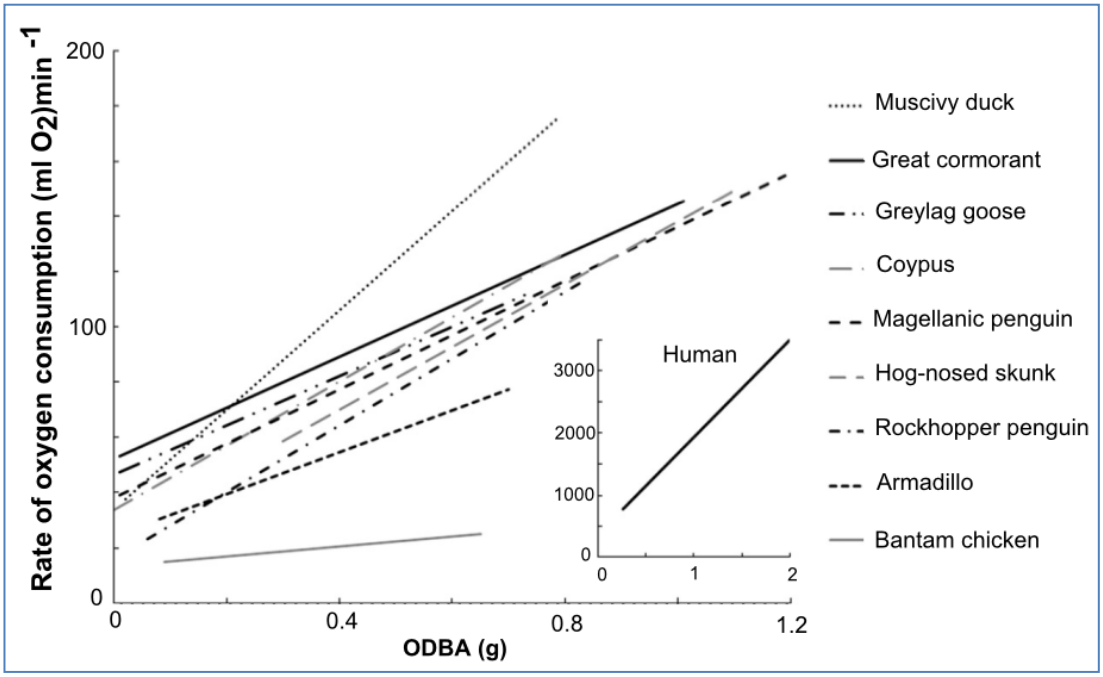


Figure 1.3 "Best fit linear relationships between rate of oxygen consumption and ODBA for a range of bipedal and quadrupedal species while resting and walking/running on a treadmill. Other behaviours were also displayed. Where data are available for multiple individuals of a species, a common slope is shown, derived from a linear mixed effects model. For clarity, the running order of species on the legend follows the order of slopes on the graph from top to bottom. Data for humans are included in an inset figure because values for rate of oxygen consumption are an order of magnitude greater than that of the other species" (Halsey et al., 2009b).

1.3 Stressed state as a limitation during the energy expenditure measurement

Furthermore, calibration experiments are arguably stressful to the subject animals (Groscolas et al., 2010) and can potentially invalidate the energetic data or the calibration relationship obtained. For instance, an excess of 81% of heart rate has been found in king penguin due to an anthropogenic stressor (Viblanc et al., 2012a), but this study did not look at the magnitude of the effect of the stressor on $\dot{V}_{O_2}$. Groscolas et al. (2010) compared his calibration (Energy expenditure- heart rate) derived from data for "unstressed" king penguins with the calibration of Fahlman et al. (2004; $\dot{V}_{O_2}$- heart rate), using a more conventional protocol and thus with potentially stressed king penguins. Groscolas et al. (2010) found an underestimation of energy expenditure (average of 25%) when using the potential stressed-biased calibration (More details in Chapter four, Figure 4.1). Finally, several studies (e.g. Groscolas et al., 2010, Green, 2011, McPhee et al., 2003) have already suggested that a stressed state independently affects the cardio- respiratory and behavioural (which modifies the accelerometric data) responses. However none of them actually measured the magnitude of the effect of the stress response on the different systems. Furthermore nothing is known about the effect of a stressor on the $\dot{V}_{O_2}$ and VeDBA in king penguins. For this reason chapter three measured the changes in heart rate, rate of oxygen consumption and levels of activity in king penguins in response to a stressor to define their stress response. Nonetheless no energetic research has so far measured the effect on a subject animal of the stressed state resulting from the experimental environment and protocol in place, nor on the ability of experimental animals to acclimate. Chapter four in particular investigates the acclimation of king penguins to the experimental environment and protocol encountered during respirometry experiments. Chapter four also looks at the bias removal concerning specifically the respiratory, cardiac and behavioural stress responses.

1.3.1 What is stress?

Stress is often described as a threat to homeostasis where homeostasis is, as defined by McEwen and Wingfield (2003), the "stability of physiological systems that maintain life" (e.g. pH, body temperature, glucose levels). Cardiac, thermic and behavioural stress responses in penguins have already been studied (Nimon et al., 1995, Culik and Wilson, 1991, Viblanc et al., 2012a, Regel and Pütz, 1997). However, stress responses are typically difficult to generalise because they can differ between species (Hill et al., 2008), individuals (Romero, 2004) due to life histories, especially during early development (Kitaysky et al., 1999b, Kitaysky et al., 1999a), over time with acclimation (Romero, 2004), between stressor types (Moberg and Mench, 2000), and when multiple stressors occur simultaneously (Dallman et al., 1992). This is also partly because stress affects the entire physiology of an organism (Hormonal, cardiac, immune system, psychology, etc.), which means that assessing and measuring 'stress' is complex. Additionally, the wording related to 'stress' is sometimes misused or unclear within the scientific community (Romero, 2004). To avoid confusion, this thesis used the definitions of Romero (2004); **Stressor**: 'a noxious or unpredictable stimulus that causes a stress response'. '**Stress response**: the physiological, hormonal and behavioural changes that enable an animal to cope with a stressor'. '**Acclimation**: the concept that after repeated or chronic exposure to a stressor, an animal no longer considers the stressor to be noxious and reduces its glucocorticoid response.'

1.3.2 Stressor

Different stressors (thermic-, toxic-, etc.) generate different physiological responses (Moberg and Mench, 2000). This thesis concentrates only on external negative stressors (i.e. fear or tension stress: predator detection, aggressive social interactions, shock avoidance, death-threatening manoeuvres, detection of human presence for unacclimated individuals, etc.). In humans, the closest equivalent would be a 'psychological stressor', but as this research is on wild animals, the term 'disturbance stressor' is implied when the word stressor is used.

Furthermore, as the reaction to human presence is being investigated, the stressor was specifically an 'anthropogenic disturbance stressor'.

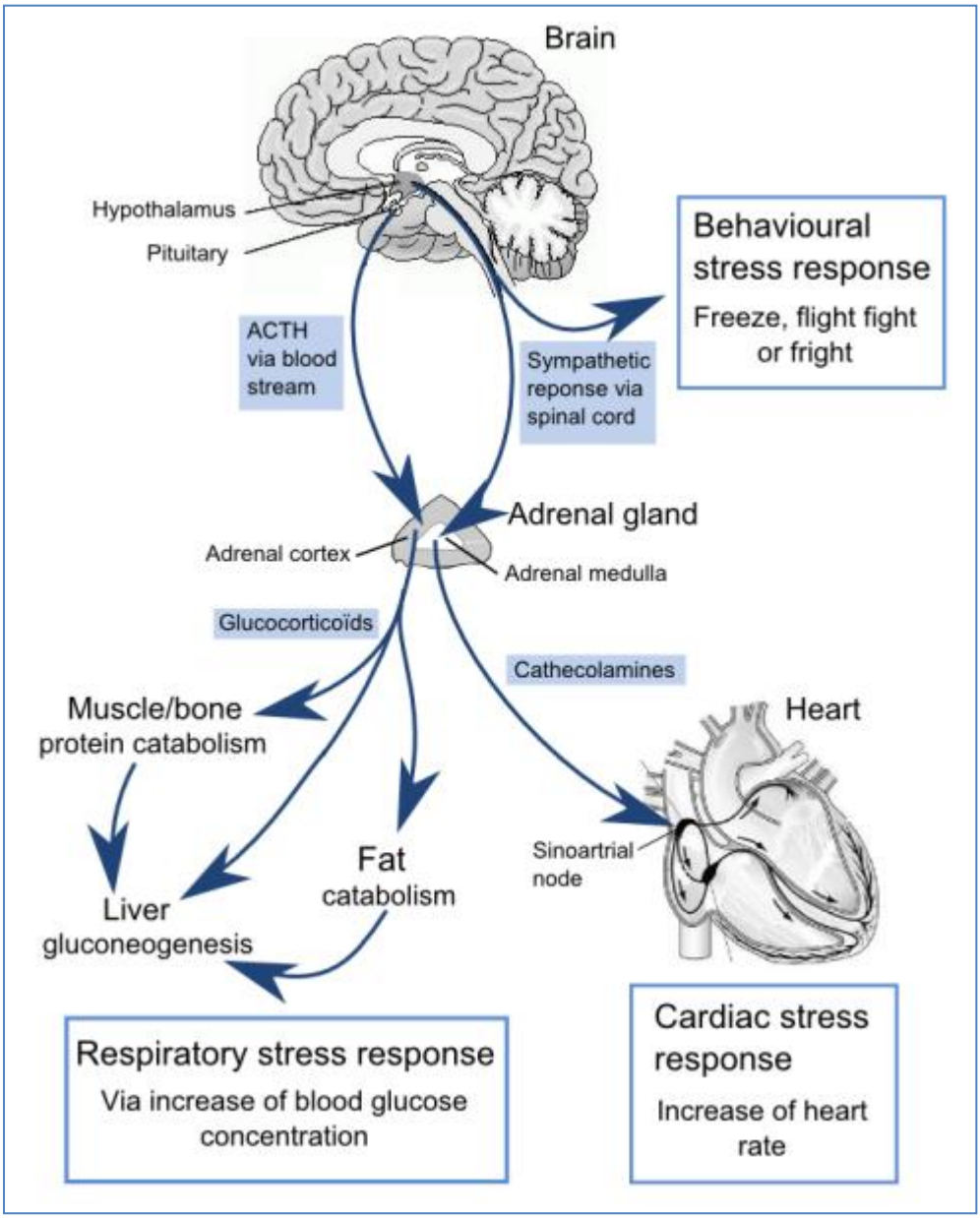


Figure 1.4 Simplified pathway showing the cardio, respiratory and behavioural stress responses. ACTH : Adrenocorticotropic hormone. Adapted from multiple sources: Romero (2004), Von Borell et al. (2007) and Moberg and Mench (2000).

1.3.3 Short term stress response

Reactions to stressors affect almost all the physiological functions of an organism via the sympathetic and parasympathetic system (Hill et al., 2008). When a stressor is perceived by the central nervous system, catecholamine is secreted and acts on the sympathetic system by increasing heart rate (von Borell et al., 2007, McCraty, 1996). With a delay of about a minute, and if the stressor is strong enough, another hormonal cascade is engendered through the hypothalamic-pituitary-adrenal axis which finally secretes glucocorticoids. This pathway enables changes in metabolic rate and $\dot{V}_{O_2}$, as the glucocorticoid response regulates blood sugar concentration, used to create ATP by aerobic respiration (Hill et al., 2008). All these changes prepare the "fight or flight" (Cannon, 1929) or the updated "freeze, flight, fight or fright" (Bracha et al., 2004) behavioural stress response. As this thesis also involves short term energy expenditure measurements, focus is on the stress responses affecting the respiratory, cardiac and behavioural systems (Figure 1.4).

1.3.4 Measuring the stressed state and subsequent acclimation

Several techniques exist to measure stressed states. Some of them require blood sampling, some are only suitable for measuring short term reactions, while others require complex knowledge or materials. The most commonly measured variables are: hormones, heart rate, vocalisations, behaviour (vigilance), and respiration. By definition, the theoretical reference method is the measurement of blood hormone (i.e. glucocorticoids) (Romero, 2004, Hill et al., 2008). As mentioned earlier, stress responses are directed by a hormonal reaction which activates/inhibits different body systems. As this thesis focuses on energy expenditure measurements, glucocorticoids were not measured, but only variables related to energy expenditure as respiratory gas exchange, heart rate and activity levels, i.e. behaviour (Figure 1.4). Indeed glucocorticoids would have led to an additional specific stress response linked with blood sampling. Consequently, the term 'stressed' data used in this thesis refers to 'data collected under an anthropological disturbance'. The 'unstressed data', representing 'acclimated data' mostly refers to 'data with stressor-induced bias removed'. However these

'unstressed data' may still represent a physiological stressed state, albeit less stressed than the experimentally induced stressed state, or during the first period after being placed in the experimental environment.

1.4 King penguins

1.4.1 General information

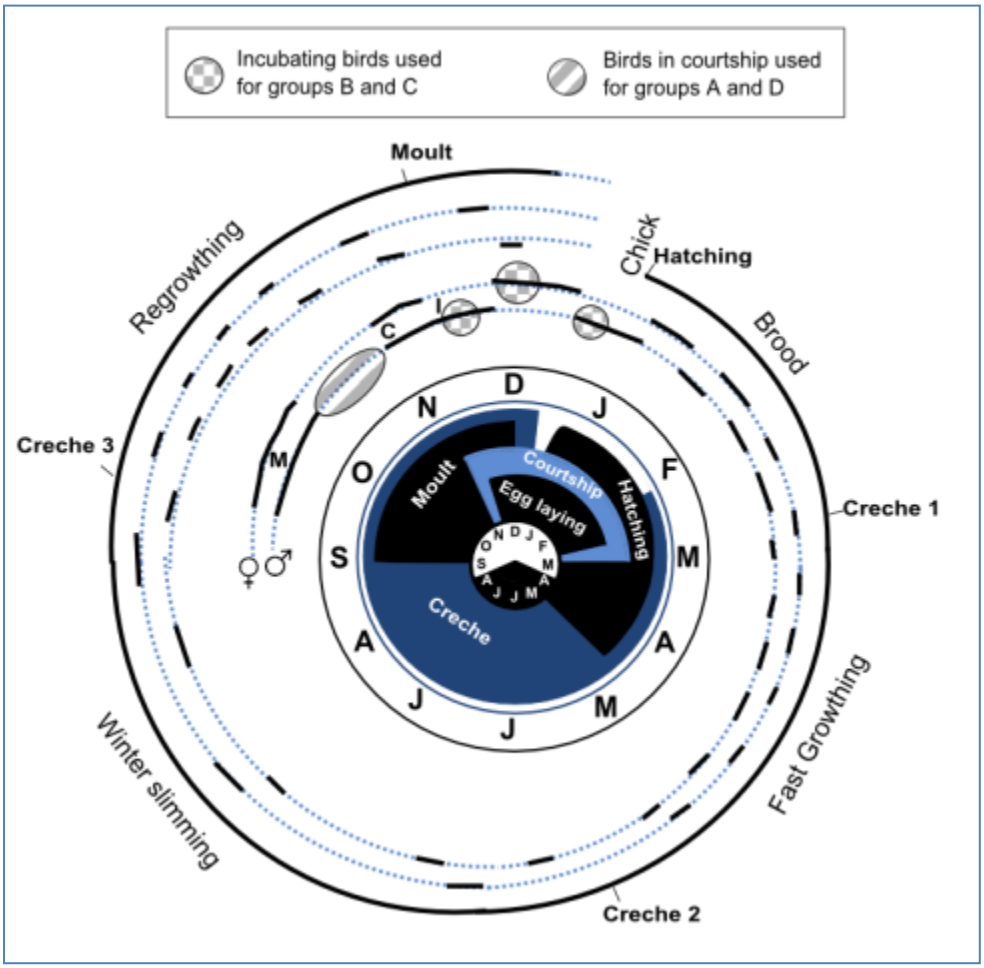


Figure 1.5 Life cycle of king penguin colony. The centre circle denotes the twelve months of the year, with the summer in white and winter in black. The surrounding pie charts describe the breeding state of king penguin colony. A repetition of the months is provided around the outside of those charts. The spirals represent the breeding cycle of a typical, successful pair of breeding king penguins. M is for moult, C for courtship and I for incubation. Plain lines represent when the bird is onshore and the dashed line represents when the bird is at sea. The striped shaded area represents the kind of individuals taken for groups B and C of this study, while the checked shaded areas represents the kind of individual taken for group A and D (Table 2-1). Illustration adapted from Ménard J.J.,(1998).

King penguins (*Aptenodytes patagonicus*) are the second largest species of extant penguin (from 9 to 16 kg at Crozet Archipelago; Barrat, 1976) after the emperor penguin (*Aptenodytes forsteri*). Both sexes look similar, but females are slightly smaller. King

penguins are pelagic (Ainley et al., 1992), spending most of their time at sea. They come onshore for breeding and moulting, forming big colonies. King penguins arriving at the beginning of the breeding season are considered as early breeders, while birds laying after mid-January are considered as late breeders (Figure 1.6). As king penguins eat mainly myctophid fish and some cephalopods (Cherel and Ridoux, 1992), they fast whenever they are ashore. Offspring care switches between partners throughout the breeding season (termed shifts). One partner stays onshore while the other is foraging at sea (Figure 1.5). Foraging trips become progressively shorter over the season from 24.0 ± 4.9 days in early November to 21.6 ± 3.0 days in mid-December at Crozet Archipelago (Barrat, 1976). Parents take turns to incubate the egg and chick with an average of five shifts over the incubation period. Figure 1.5 summarises a successful reproductive year for a breeding pair of king penguins. Most colonies are situated near the shore, but some can be further inland as on Ile aux Cochons, Crozet Archipelago (Barrat, 1976). This colony is 1.3 km inland and at an altitude of 100 m. The size of a colony can reach 200 000 pairs (at Ile aux Cochons) with a mean distance of 89 cm between eggs (at Crozet and Kerguelen Archipelagos; Bauer, 1967). King penguins have no nests but have a so-called 'zone of attachment' (Barrat, 1976). The single egg is incubated on the feet of the adult in a special 'brood pouch' (Handrich et al., 1995), the incubating parent being able to walk a short distance if necessary, as, for example, to escape water flooding.

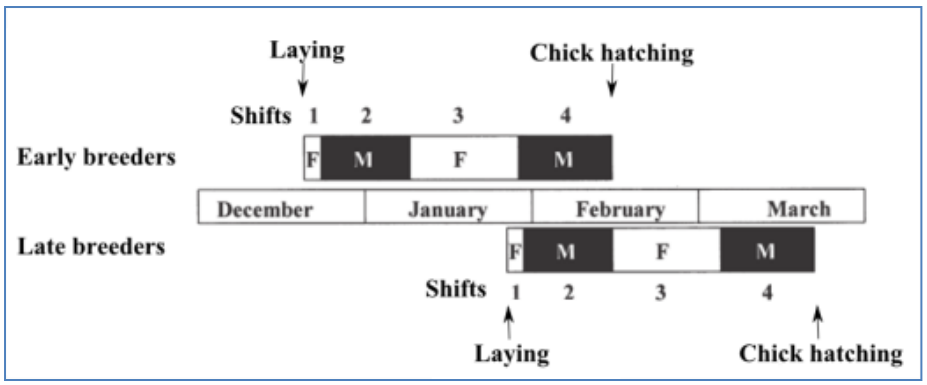


Figure 1.6 Early and late breeder' incubation shifts in relation to the month. F = female; M = male (modified from Gauthier-Clerc et al., 2002).

1.4.2 Actual population state

The potential deterioration of resources in the Southern Ocean may increase the sensitivity of long-lived, diving predators such as penguins (*Sphenisciformes*) to energy-dependent factors. A better understanding of their energy costs would provide insights into their energy budgets and its limits. This would improve their protection by focusing on specific sensitive areas. Penguin populations are considered to be vulnerable to environmental changes and to exhibit slow recoveries in case of population crashes (Williams, 1995). Many species of penguin breed in a relatively limited latitudinal range, whether that is within the subtropics, the Antarctic, or more temperate latitudes. These birds, towards the top of the food chain, are good indicators of the effect of environmental change in ecosystems (Aebischer et al., 1990, Gjerdrum et al., 2003, Voigt et al., 2003, Halsey et al., 2007b, Williams, 1995). Indeed, they depend on the rich southern marine area, and are sensitive to any changes in the abundance and distribution of their prey (Williams, 1995). In addition, recent climatological changes and other anthropogenic influences may result in lowering survival success of king penguins. For example, Peron et al. (2012) have predicted an extension in foraging trips for king penguins within the next decade as the polar front will move further south due to global warming. Overfishing has also been shown to impact on penguin populations (Williams, 1995, Burger and Cooper, 1984). While at sea, king penguins spend most of their time on the surface, which increases the likelihood of their contact with pollution (plastic, oil etc.) (Williams, 1995). The breeding cycle of penguins is long and is associated with low reproductive success (only 21.5% of the birds returning at the colony of Baie du Marin successfully raised a chick in 1998-2000; Descamps et al., 2002). For example, king penguins attain sexual maturity at the age of four and lay only one egg per attempt. Their breeding cycle lasts more than a year, precluding the possibility of reproducing successfully every year (Williams, 1995). In recent years, colonies of king penguins are typically increasing (Weimerskirch et al., 1992, Woehler and Croxall, 1997, Woehler et al., 2001,

Delord et al., 2004), probably recovering from hunting by sealers during the 19th century (Delord et al., 2004). However, since the 1990's, further anthropogenic impacts have served to blight penguin populations, for example the observed decreases of some colonies of Adélie (*Pygoscelis adeliae*) and chinstrap (*Pygoscelis antarcticus*) penguins (Culik and Wilson, 1991). Explanations focused on the possibility that the birds were experiencing increased stress levels due to, for example, increases in tourism (Culik and Wilson, 1991, Nimon et al., 1995, Culik and Wilson, 1995). Indeed, being a relatively curious, large, flightless bird living in colonies makes this species fairly easy to approach. Interestingly, all the colonies of king penguins on Possession Island (one of the five Islands in the Crozet Archipelago) are increasing, resulting in an overall increase in the island's population (Figure 1.7), with the exception of the colony at La Baie du Marin (Figure 1.8) (Delord et al., 2004). La Baie du Marin is the only colony subject to an almost constant anthropogenic presence, occasionally used for boat access, as well as being entered daily for scientific research throughout the year. Research into anthropogenic effects on penguin populations has increased in recent years as a rise in studies into the effects of disturbance such as the effect of certain markers or loggers that disturb the hydrodynamic shape of penguins (Saraux et al., 2011, Gauthier-Clerc et al., 2004, Jackson and Wilson, 2002, Hindell et al., 1996, Bannasch et al., 1994). Flipper bands extend the king penguin's foraging trips due to a decrease in their foraging efficiency, which leads to a decrease in adult survival and chicks successfully reared (Saraux et al., 2011). Moreover, disturbance resulting from direct human presence (Culik and Wilson, 1991, Nimon et al., 1995, Culik and Wilson, 1995, Viblanc et al., 2012a, Nimon et al., 1996) has also aroused interest. However, while measurements of heart rate have already demonstrated a stress response (Nimon et al., 1996, Nimon et al., 1995, Viblanc et al., 2012a), as yet no studies have looked directly at the short-term cost of this disturbance. For this reason, chapter three investigates the effect of a stressor on the energy expenditure of king penguin, via $\dot{V}_{O_2}$, heart rate and levels of body movement.

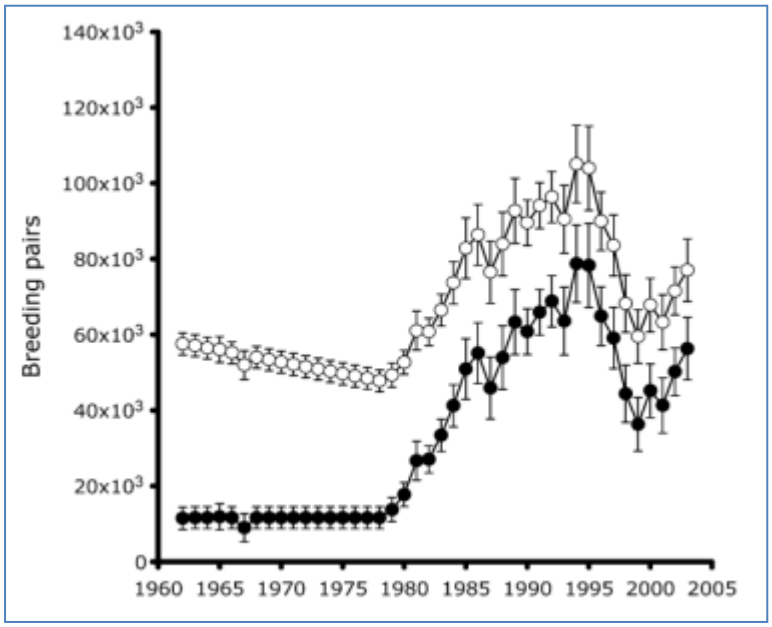


Figure 1.7 Estimates of the breeding populations of king penguins on Possession Island, Crozet Archipelago. White circles: estimates are computed for all the colonies of the island combined; Black circles: estimates were computed excluding the colony of la Baie du Marin. Errors bars indicate ±SE (from Delord et al., 2004).

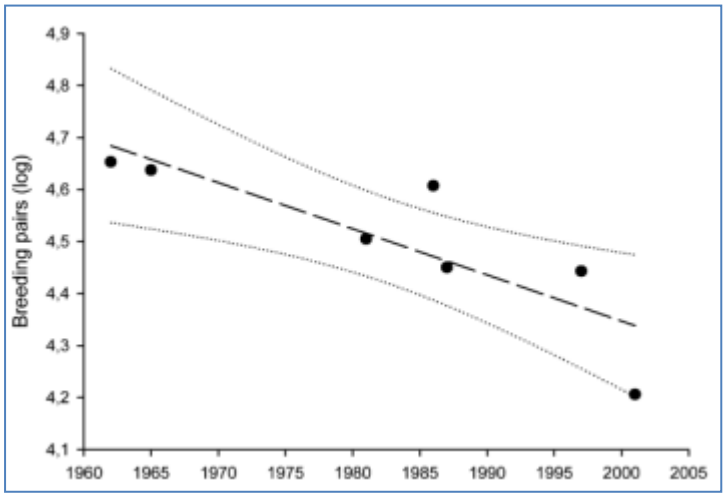


Figure 1.8 Population model fitted to counts of breeding pairs (logged) 1962– 2003, for the colony of la Baie du Marin, Possession Island. Lines indicate predictions of linear regression models (dashed line), including 95% of confident intervals (dotted line) (from Delord et al., 2004)

1.4.3 King penguin energetics and ecology

The life cycle of penguins, including extreme meteorological conditions, attracted attention and studies on their energy expenditure (Nagy et al., 1984, Wilson et al., 2004). Their skills as air-breathing deep divers attract research interest into their at-sea energy expenditure, indeed they spend most of their time in water and swim considerable distances during foraging trips. Several studies have estimated their energy expenditures at sea or their levels of energy intake (Kooyman et al., 1992, Hanuise et al., 2010). Their restricted energy expenditure budget while on shore has also attracted attention (Halsey et al., 2007b, Gales and Green, 1990, Halsey et al., 2008a, Groscolas et al., 2010, Viblanc et al., 2012a, Viblanc et al., 2011a, Fahlman et al., 2005, Brown et al., 2004, Groscolas, 1990, Groscolas et al., 2007, Groscolas and Robin, 2001, Halsey et al., 2008c). Indeed while on shore king penguins are fasting as they only eat at sea. Consequently, the success of their reproduction, as well as for individual or species survival, depends on effective management of energetic expenditures during fasting periods (Brown et al., 2004, Viblanc et al., 2011a, Groscolas, 1990, Groscolas et al., 2007, Groscolas and Robin, 2001, Halsey et al., 2008c). During the reproductive season king penguins spend 30 to 50% of their time onshore (successful and unsuccessful reproduction, respectively, Descamps et al., 2002). The pair shares the parental care of their chick. Thus, parents are submitted to intermittent fasting periods (an average of 10 egg or chick exchanges occur between a pair of breeding adults) and foraging periods at sea. While onshore, they stay protecting the egg or the chick, and while foraging, they hunt close to the polar front in the open sea. The beginning of each foraging trip requires the parent to walk from the ‘zone of attachment’ (Barrat, 1976) (King penguins have no nests) to the sea or vice versa. However, some king penguins nest more than two kilometres from the sea (Guinet et al., 1995, Halsey et al., 2007b) and must walk this distance either laden after foraging or having fasted for up to a month. Halsey et al. (2007b) estimated walking energy expenditure by measured $\dot{V}_{O_2}$ at different speeds and body masses in king penguins. Interestingly, even though total weight loss may have represented a third of their maximum

(i.e. sated) body mass, according to these treadmill studies, heavy king penguins used around the same amount of energy to move a unit distance as when much lighter having fasted (Halsey et al., 2007b). This showed an optimised adaptation of the cost of load carrying, contrasting with observations within other species, including humans (Browning et al., 2006, Marsh et al., 2006, Griffin et al., 2003, Tickle et al., 2010, Taylor et al., 1980) where the cost of transport was higher for heavier subjects. As the factors influencing the energy expenditure of pedestrian locomotion are still unclear, investigating this optimised adaptation to understand its mechanism could enable a better understanding of the factors influencing the cost of transport. Chapter five looks at the energetically 'optimised fat penguin' using biomechanical measurement through tri-dimensional analyses of the gait of king penguins, as well as their global triaxial accelerometry.

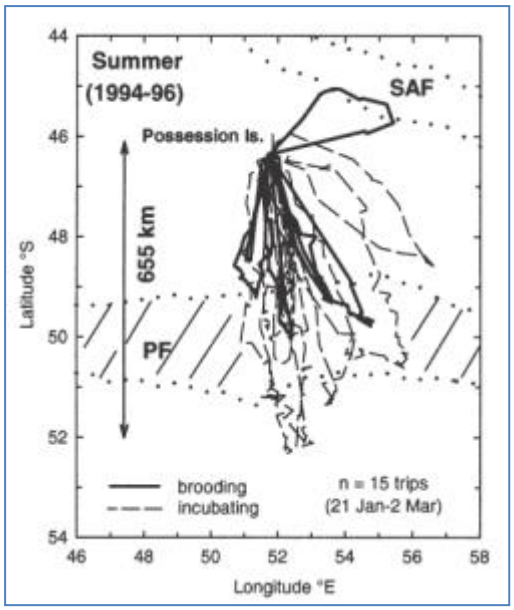


Figure 1.9 Tracks of king penguins from Possession Island (Crozet Archipelago), instrumented with GPS during the summer (incubating and brooding stages). PF is for the Polar front and SAF is the sub-Antarctic front (from Charrassin and Bost, 2001).

Halsey et al (2007b) concluded that the cost of pedestrian locomotion of king penguins may not be submitted to selection pressures since even a long walk of two kilometres to the colony represented only an additional increase of 1% in $\dot{V}_{O_2}$ for the entirety of a 20 days sojourn offshore. However these calculations did not account for parameters for which

relevant measurements had not yet been recorded such as topography and stressed state (e.g. due to conspecific interactions between breeders within the colony; (Williams, 1995). Due to the predicted extension in foraging trip (Peron et al., 2012), the key moment of the longest fasting period (involving the courtship until the second egg shift, which is endured by the male; see Figure 1.5 for king penguin reproductive cycle) could become a sensitive limiting factor of the reproductive success of king penguins. A male can fast for a period of 29 days from courtship until the second egg shift, at which point it is finally able to go foraging (Gauthier-Clerc et al., 2001). However, the male still has to walk back to the sea from the 'zone of attachment' (which can involve a two kilometre journey) and then needs to swim out to the polar front (a journey of over 600 km south) to find its prey (Charrassin and Bost, 2001, Peron et al., 2012) (Figure 1.9). Thus an extension of the foraging trip due to global warming would involve a supplementary cost to their restricted energetic budget. Additionally, this long fasting period depends on the female returning from its foraging trip to the polar front. Thus an extension in foraging trip duration would lead to an extension in fasting period for the partner. Consequently, small factors influencing energy expenditure such as walking on an incline and while stressed could be decisive factors in modelling the onshore energy expenditure of king penguins. The energy expenditures of incubating birds have already been studied by Viblanc et al. (Viblanc et al., 2012a, Viblanc et al., 2011a, Viblanc et al., 2012b) resulting in an estimation of the cost of each behaviour (such as cleaning, defence, etc.). However the additional cost of stress response *per se* has not been taken in account. Chapter six estimates the cost of this longest fasting period as an example of using energy expenditure to better understand the ecology of a species. A simple estimation compared the energy expenditures of two groups of breeders (i.e. early and late, which are known to have very different reproductive successes; Descamps et al., 2002) while incubating, taking the cost of the stress response of territory defence into account, as

well as the cost of walking to the zone of attachment over terrains representing different inclines.

1.5 Aims of the thesis

This thesis has four aims focussed on providing a better understanding of the onshore life of king penguins, especially their physiological stress response and the biomechanics of their pedestrian locomotion, using their energy expenditures. One aim is addressed per chapter. The aims are (1) to assess the cardio-respiratory and behavioural stress responses and their cost in king penguins, accounting for the movement (**chapter three**), (2) to avoid stress-induced errors in estimates of energy expenditure by defining an appropriate protocol ensuring acclimation of the king penguin to the environment and protocol of calibration experiments (**chapter four**). (3) To find the parameters influencing the energy expenditure of walking, using the optimised fat king penguins as a model (**chapter five)**. (4) To estimate the cost of incubating for early and late breeders during the longest fasting period, taking the cost of stress response and walking on an incline into account (**chapter six**).

2.General Methods

2.1 Material and methods

2.1.1 Preparation and fieldwork

During two austral summers, fieldwork was undertaken within the king penguin colony at 'Baie du Marin' (Figure 2.1) on Possession Island, Crozet Archipelago, Southern Indian Ocean (46°25'S; 51°52'E). The first summer campaign ran from November 2009 to March 2010, while the second campaign ran from November 2010 to March 2011. Due to the remote location of the island (both geographically and in term of communications), scientific and non-scientific preparations (e.g. health check, visa application) prior to the trips were substantial. Planning of experiment and scientific approval had to be obtained two years prior, and one year prior for ethics approval. Materials had to be prepared and sent two months in advance from the French National Centre for Scientific Research in Strasbourg, France. The journey to reach the island involved a flight to the island of La Reunion, then a four day trip by ship (the Marion Dufresne) to Possession Island (Figure 2.2). Transfer to the island from the ship was via helicopter, which also transported all supplies from the ship to support the functioning of the island for the next few months. The Marion Dufresne typically services Possession Island four times a year, in November, December, March and August. The respirometry chamber custom-made for this project was not produced sufficiently in advance such that it could be shipped to Possession Island for the first field season. For this reason the first summer campaign focussed predominantly on developing and trialling experimental designs and methods.

Figure 2.1 Baie du Marin colony and the laboratory shelters (Courtesy of Maxim Loubon).

Life on the island involved some specific administration, rules (i.e. need to know first aid, rudiments of fire fighting) and social life on Possession Island, which sometimes do not prioritise research (i.e. experiment were not allowed while in the presence of visitors). Consequently, the first season helped me to acquire all the knowledge and skills needed for independent work, as well as to select the best place for the experiment and the best set up for the video recording. For the second season, the video recording protocols were improved, as this equipment had never been used in such rudimentary conditions, and thus needed to be thoroughly tested. All other techniques have already been used on king penguins, thus no tests were needed but a protocol to use all techniques simultaneously needed to be developed.

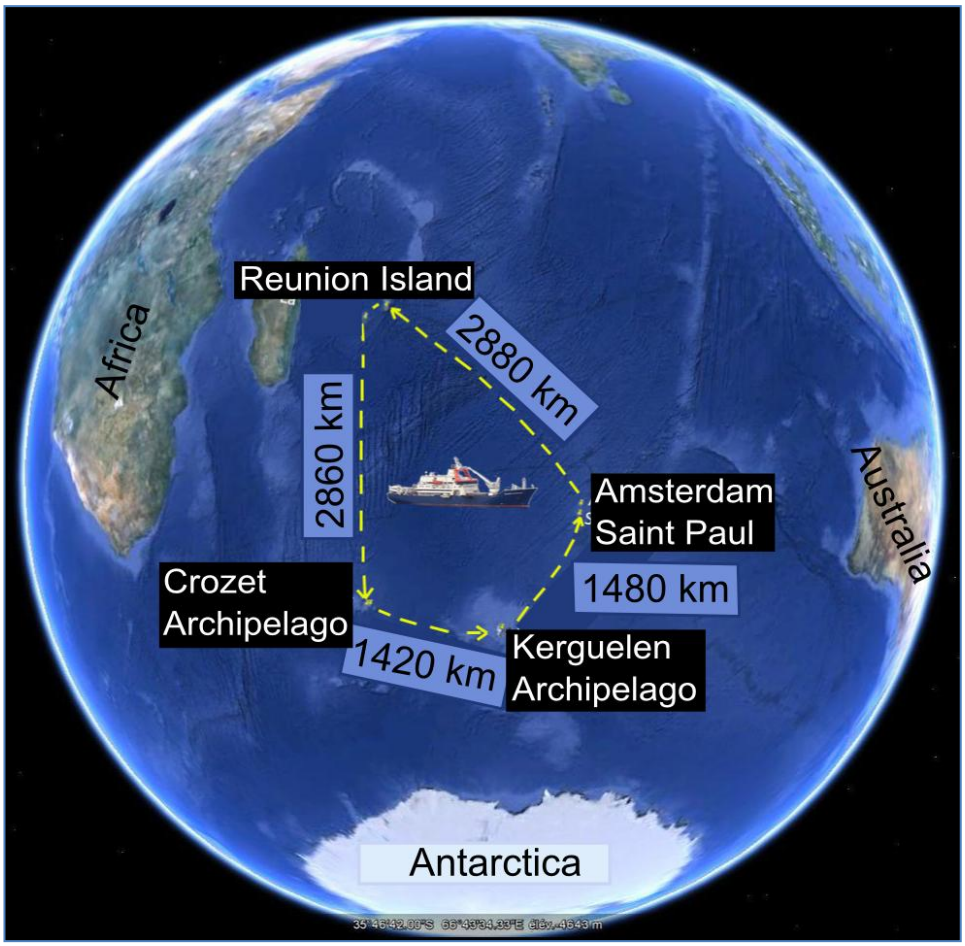


Figure 2.2 Journey of the Marion Dufresne (picture adapted from http://www.marine-marchande.net/Petits_Reportages/Auroy/Marion/00-Rotation,.jpg).

2.1.2 Ethics

All procedures used in the present study were approved by the Ethical Committee of the Institut Polaires Français-Paul Emile Victor (IPEV) and the Ministère de l'Environnement, as well as the ethics committee of the University of Roehampton. The requirements of the United Kingdom (Scientific Procedures) Act 1986 were followed. Guidance to researchers based in the United Kingdom using similar methods was used as reference.

2.1.2.1 Subject birds

Thirty two birds were used in experiments during this project (10 during the first field season; 22 during the second). The bodies of two dead penguins were also measured. Details about the selection of the subject birds and the experimental protocol are explained only for

the birds for which the data were subsequently analysed. Table 2-1 is a summary of the different experimental groups of birds, the experimental protocol and the related chapters where the data have been used.

Table 2-1 Summary of the groups of birds studied.

Gr-oup	Ex-peri-ment	Nb of birds	Sex	Reproduc-tive status (Figure 1.5)	Specification	Data collected	Used in chapter
A	Pilot	10	Male	In courtship	Heavy, periphery of the colony	Heart rate, accelerometry, body mass, video.	Pilot testing and protocol develop-ment
B	I	6	Male	In courtship	Periphery of the colony	$\dot{V}_{O_2}$, heart rate, accelerometry	3,4,6
C	I	6	Unknown	Incubating	Close to edge, low success	$\dot{V}_{O_2}$, heart rate, accelerometry	3,4,6
D	II	10	Male	In courtship	Heavy, periphery of the colony	$\dot{V}_{O_2}$, heart rate, accelerometry, body mass, video.	5,6
E	III	2	Unknown	Dead	One heavy, one thin	Centre of mass, body mass	5

Group A: The group of birds captured during the first field season consisted of ten healthy males in courtship (Figure 1.5) with high body masses (>12 kg) and able to walk on a treadmill. Selecting birds in courtship assures that they are not presently breeding. In addition, as both sexes look similar, selecting birds engaged in courtship enabled identification by behaviour (Barrat, 1976) and selection of males only, as crucial differences in physiology have been found between sexes of the same species (e.g. energy expenditure-heart rate relationships; Green, 2011). Furthermore it is assumed that birds in this state have empty stomachs and thus are post-absorptive (Halsey et al., 2007b, Gauthier-Clerc et al., 2000). This is important to ensure that the source of ATP production is mostly on lipids, enabling an accurate conversion of $\dot{V}_{O_2}$ into calories. These birds were used to develop an

experimental protocol involving the simultaneous measuring of cardiac function and gait (by accelerometry and video) while walking on a treadmill.

Group B: Six males in courtship (Figure 1.5) were captured near the shoreline at the edge of the colony. Birds were tested for their ability to walk on a treadmill and trained to do so during at least two sessions of walking, each for approximately 10 minutes.

Group C: Six relatively newly incubating birds (Figure 1.5) of unknown sex and with low likely reproductive success (late breeders nesting in a peripheral area of the colony; Barrat, 1976) were selected. King penguins taking care of a chick or incubating an egg close to hatching tend to have full stomachs. Using newly incubating birds allowed the assumption that these birds were solely metabolising lipids (Gauthier-Clerc et al., 2000).

Group D: Ten king penguins in courtship (Figure 1.5) with heavy body masses (>12 kg) were identified as males from their behaviour (Barrat, 1976), and were captured near the shoreline at the edge of the colony. They were kept for 22 days in a pen while they fasted, thus enabling data to be collected from the same individual at different body masses. They were tested for their ability to walk on a treadmill and trained to do so during at least two sessions of walking each for approximately 10 minutes.

Group E: Bodies of two dead king penguins with different body masses (13.6 and 11.1 kg) were collected from la Baie du Marin. The heavier cadaver was obtained from another research project and represented an individual that had not survived experimental surgery, thus it had a healthy body condition including substantial fat reserves. The lighter cadaver was a bird found dead within the colony and had less endogenous energy reserves. Both were frozen (-40°C) and brought back to the National Natural History Museum of Paris. The heavy cadaver was kept in alcohol (Formaldehyde and Ethanol 70%) while the light one was preserved in a frozen state (-40°C).

2.1.3 Shelters and pens

Two shelters and three pens located within the study colony (Figure 2.1) were employed during the experiments. During the first season, all experimental setups were placed upon a wooden platform to reduce the impact of the shelter on local moss ecology and reduce the ambient humidity in the shelter. However other human activities near the shelters caused vibrations which unduly disturbed the quality of the video footage obtained (§ 2.1.8.2). Thus for the second season, an alternate shelter was used for experiments, which was built into the ground. A separate shelter was employed for housing the computers used for loggers programming, equipment charging, minimising disturbance to the subject birds. The door of the first was always left open for aeration and to ensure that conditions inside were similar to those outside. When needed, two to three birds were kept within a pen. The pens consisted of wooden walls (2 m high, 3*3 m surface) with a door and with wire netting across the top to keep skuas (*Stercorarius antarcticus*) out, while enabling access to rain water. Apart from the relative lack of wind, meteorological conditions in the pens were similar to those of the colony. A thin gap in the wall enabled the monitoring of the birds without disturbance.

2.1.4 Treadmill

A treadmill (DOMYOS model TC 530, 1370 * 1520 * 782 mm) adapted to be water and faeces resistant was used during both seasons. During some experiments the treadmill was elevated using wooden planks to reach an incline of approximately 13%. The speed range was from 0.8 km/h to 16 km/h by increment of 0.1km/h and was independent of the body mass of the walker. The speeds used to collect data were: 1.0, 1.2, 1.4 and 1.6 km/h.

2.1.5 Respirometry

2.1.5.1 Respirometer chamber

The chamber (80*50*70 cm, 280 L) enabled the king penguin to comfortably walk with a natural gait. The chamber was made of translucent acrylic with six fans on the top to maintain good homogeneity of gaseous concentrations. The chamber was secured over the tread of the treadmill (Figure 2.3). Exterior air was drawn from a tube connected to the

outside of the shelter. However the system was an open-flow system as a small gap existed between the chamber and the treadmill frame, from which a small portion of the air from the laboratory entered. To minimise the gas resultant exchange through this aperture, brushes were added as skirting, around the bottom of the chamber frame. During the 'stressor experiments', because the researcher was in close proximity to the chamber, the researcher breathed into a mask incorporating a unidirectional valve such that all expired air was evacuated to the outside and thus did not affect respiratory gas levels in the laboratory.

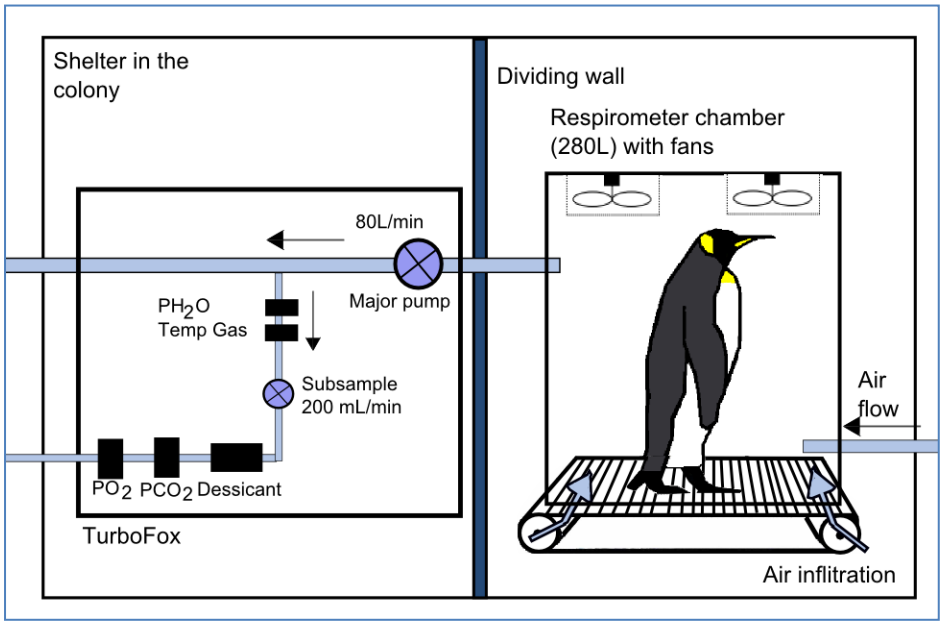


Figure 2.3 General respirometer setup and plumbing. P stands for partial pressure.

2.1.5.2 Respirometry protocol

Flow rate (FR), water vapour pressure, barometric pressure, and molar fraction of CO_2 and O_2 were measured at 1 Hz in a sample of excurrent flow from the respirometer chamber using an open-flow respirometry system (Figure 2.3), and recorded using computer software (Expedata; Sable Systems International, USA). Chosen flow rate was based on the size of the chamber: The large volume of the chamber needed a high flow rate to enable a short time constant such that the subject need not walk for an extend period until respiratory gas levels in the chamber reach equilibrium (Lighton, 2008). Additionally, a high flow rate is also

needed in open-flow systems to avoid gas leak. However a high flow rate decreases the difference between the intake and output gas concentration, reducing the measurement signal. Finally choice of flow rate needs also to consider the potential accumulation of CO_2, which, at high concentration can be dangerous for the animal. Considering all these compromises, a flow rate of 80 L min^{-1} was used. Consequently the time constant of the system was 3.5 min ($\frac{Volume}{Flow\ Rate} = \frac{280}{80}$ = 3.5, thus 3*3.5= 10.5 minutes to reach 95% equilibrium; Lighton, 2008). A sub-sample (200 mL min^{-1}) of excurrent gas was analysed by a Turbo FoxBox respiratory gas analyser (Sable Systems International, USA). Water was removed from the air by passing it through Drierite prior to analysis. The analyser was calibrated every second day (with a mixed gas of CO_2 (~1.0 %) and O_2 (19.98 %)) followed by nitrogen injection tests to validate the system (Lighton, 2008, Lighton and Halsey, 2011). The O_2 sensor was spanned each day with dry ambient air at 20.95%. The zero point of O_2 was fixed by the manufacturer and did not require calibration. The calibration of water vapour pressure was also completed every two days. The main flow of excurrent air from the respirometer was evacuated to the outside. During the experiment, baselines from the outside air were taken approximately every half hour to measure the drift of the sensors due to changes in temperature and pressure throughout the day.

2.1.5.3 Respirometry calculations

Expedata was used to correct spikes and analyser drift in measurements of O_2 concentration. To interpret near instantaneous changes in O_2 concentration in the chamber, the 'instantaneous equation' defined by Woakes and Butler (Woakes and Butler, 1983) was used to calculate volumes of O_2 uptake (V_{O_2}, in ml) between any two points in time (i.e. t_1 and t_2, in sec) of fraction of O_2 (F_{O_2}) in time (as in Halsey et al., 2009b).

Equation 2-1

$$V_{O_2(t_2)} = \left[F_{eO_2(t_1)} - F_{eO_2(t_2)}\right] * V + \dot{V}_c * (t_2 - t_1) * \frac{\left[2F_{iO_2} - F_{eO_2(t_1)} - F_{eO_2(t_2)}\right]}{2}$$

;where t_1 and t_2 are two points in time (in sec) with their respective fraction of O_2 (F_{O_2}),V is the chamber volume in ml, $\dot{V}_c$ is the dry flow rate (corrected for removed water vapour) in ml min^{-1} and the subscripts 'e' and 'i' are for excurrent and incurrent F_{O_2}, respectively. The instantaneous equation defined by Woakes and Butler (1983) assumes RER to be close to 1 and invariable throughout the activity. For this reason, CO_2 can be replaced in the equation and the final equation only includes O_2. It is assumed that birds used in the present study were only metabolising lipids, as they were fasting. They were thus assumed to have empty stomachs and therefore post-absorptive (Halsey et al., 2007b, Gauthier-Clerc et al., 2000). However, RER was checked to be lower than 1 and reasonably constant before the analyses. Rate of O_2 consumption per minute ($\dot{V}_{O_2}$, in ml/min) was found by multiplying $V_{O_2(t_2)}$ by 60.

2.1.6 Heart rate

The make of heart rate data loggers used during this project (models RS400, RS800 or RS800lite, Polar Electro Oy, Kempele, Finland) have been used previously in several research projects on king penguins (Viblanc et al., 2012a, Viblanc et al., 2011a, Groscolas et al., 2010). The heart rate logger consisted of a cardiac electrical activity detector and emitter, as well as a watch receiving and saving the data. The emitter filtered the electric signals received by both electrodes enabling it to accurately calculate heart rate without bias from other electrical activity such as due to muscle contraction or electrode noise. Data were transferred instantaneously to the receiver by radio-wave transmission. Adaptations of this human heart rate logger were made following Groscolas et al. (2010), consisting of the addition of security pins to the extremity of the emitter. The logger was attached using adhesive tape (Tesa® 4651) to the middle of the subject bird's back to avoid hindering natural movements (Figure 2.4). Heart rate was calculated and recorded each second. Once the equipment was removed, data were transferred from the Polar watch onto a computer using the Polar logger software: output files were then cleaned for outliers and subsequent data analysis conducted with R Cran (R Core Team, 2012).

Figure 2.4 View of a Polar attached to a king penguin. Polar emitter (left) and a Polar receiver (right).

2.1.7 Accelerometry

Acceleration data loggers (Figure 2.5) were attached to the feathers of the penguin with tape (Tesa® 4651) on the bird's back, at the height of the hip (Figure 2.6). This is the assumed height of the centre of mass, which is the unique spatial point where the weighted relative position of the mass distribution sums to zero. Data were recorded at 32.5Hz. The accelerometers were made by the Département Ecologie, Physiologie et Ethologie (DEPE, department of the IPHC, CNRS, Strasbourg), model Macrologger FCM (Medina, R. Laesser, Strasbourg, France, 85*35*18 mm, 80g.). The triaxial accelerometers (3*3 mm) were made of three accelerometry sensors fixed perpendicularly. Each of the sensors is sensitive to both the acceleration due to the Earth (gravitational acceleration) and the changes of speed of one body axis, i.e. body accelerations, whose acceleration resultant is perceived and measured as raw acceleration of the given axis.

Figure 2.5 Accelerometer data logger being attached to a penguin.

Figure 2.6 Position of the loggers. The accelerometer data logger is placed on the backbone. The heart rate data logger is placed higher on the right side of the back

A simplified schematic view of an accelerometer is as a weight held by two springs, one at each end, able to move in only one axis in a cylinder as illustrated in Figure 2.7 and Figure 2.9. The recorded raw accelerations for a given axis is the sum of two components: the static (static body acceleration, SBA) and the dynamic accelerations (dynamic body acceleration, DBA). The static body acceleration measurement represents the orthogonal projection of the reaction to gravitational acceleration on the given axis (Figure 2.8) and is included between [-1 g, 1 g] (g = 9.81 m/s^2). The general equation is a scalar production (Equation 2-2). The norm of the resultant of the three static body accelerations is equivalent to 1 g. Because the gravity vector is always and strictly orientated on the vertical, the three static body accelerations directly vary with body orientation (i.e. posture) and its change (i.e. tilt angles as pitch and roll. Further descriptions on the angles in § 2.1.7.1). For example, the horizontal position of the accelerometer illustrated in Figure 2.8 represents a static body acceleration of 0 g of the axis x, while an upright vertical position leads to a static body acceleration of -1 or +1g depending of the direction. To simplify the interpretation of the position of a logger, signs of the results of the scalar production were transformed to represent the displacement of the logger and not the reaction to inertia. For instance, when the logger is in an upward position, the resulting acceleration is positive (as in Figure 2.8), even though the mathematical scalar production, i.e. the measured acceleration, is negative.

Equation 2-2

$$\overrightarrow{a_s} = \|\vec{g}\|(\cos \propto)$$

; where $\overrightarrow{a_s}$ is static body acceleration, $\vec{g}$ is gravitational acceleration and $\propto$ is the angle between the axis of measurement and $\vec{g}$.

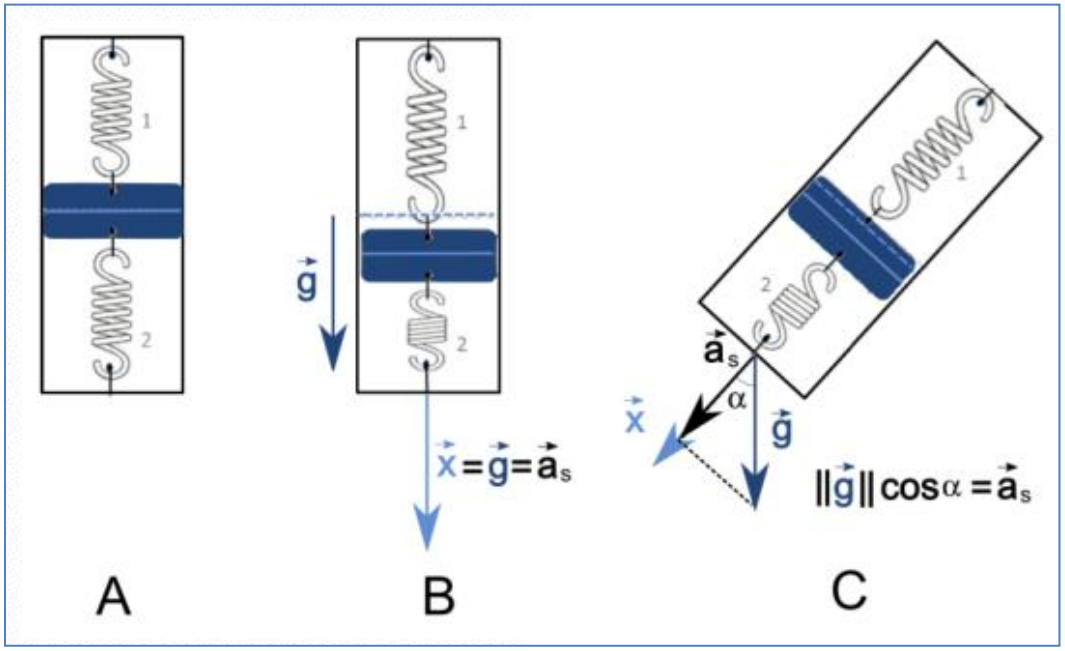


Figure 2.7 Representation of static body acceleration. Effect of the orientation on an accelerometer, schematically represented as a weight pulled by two springs, one on each side, able to move in only one axis in a cylinder (Axes called x in this illustration $\vec{x}$). In the three examples, the device is immobile. **A.** The device is not submitted to any acceleration, not even gravitation ($\vec{g}$). **B.** The device is vertical and submitted to gravity, thus the measured scalar production of static body acceleration ($\vec{a_s}$) is equivalent to gravitational acceleration ($\vec{g}$). **C.** The device is inclined on the right with an angle $\propto$ thus $\vec{a_s} = \|\vec{g}\|(\mathbf{cos} \propto)$ (Adaptation of Figure by Yves Handrich).

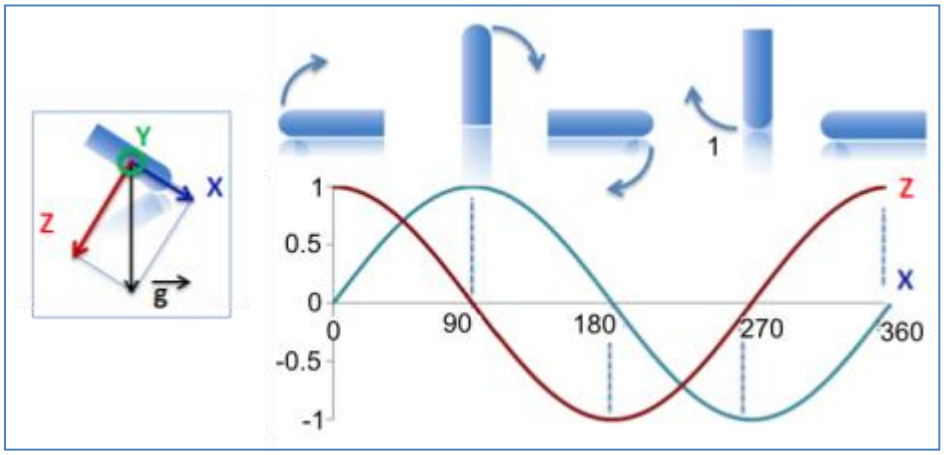


Figure 2.8 Graphical representation of orientation of an accelerometer and its related static body acceleration measured on the x and z axes (Adaptation of Figure from Yves Handrich). Note that the scalar production of x when in an upward vertical position should be -1g, however to simplify interpretation, the sign of the acceleration weas transformed to represent the direction of the displacement of the logger and not the reaction to the inertia of the movement (See text).

The dynamic component represents the orthogonal projection of the resultant change in velocities (i.e. inertia) of a movement on the given axis with the effect of gravitational

acceleration removed. However if no changes of velocity occur within the movement (e.g. static position or constant velocity), the accelerometer only measures the gravitational acceleration, as in Figure 2.7. Fortunately, in animal propulsion, even if global velocity remains constant, the displacement always corresponds to an alternation of accelerations and decelerations resulting from the propeller's activity (wings, flippers, legs, limbs) and the continual loss of energy to the environment (e.g. drag effect). This permanent change in velocity corresponds to body acceleration.

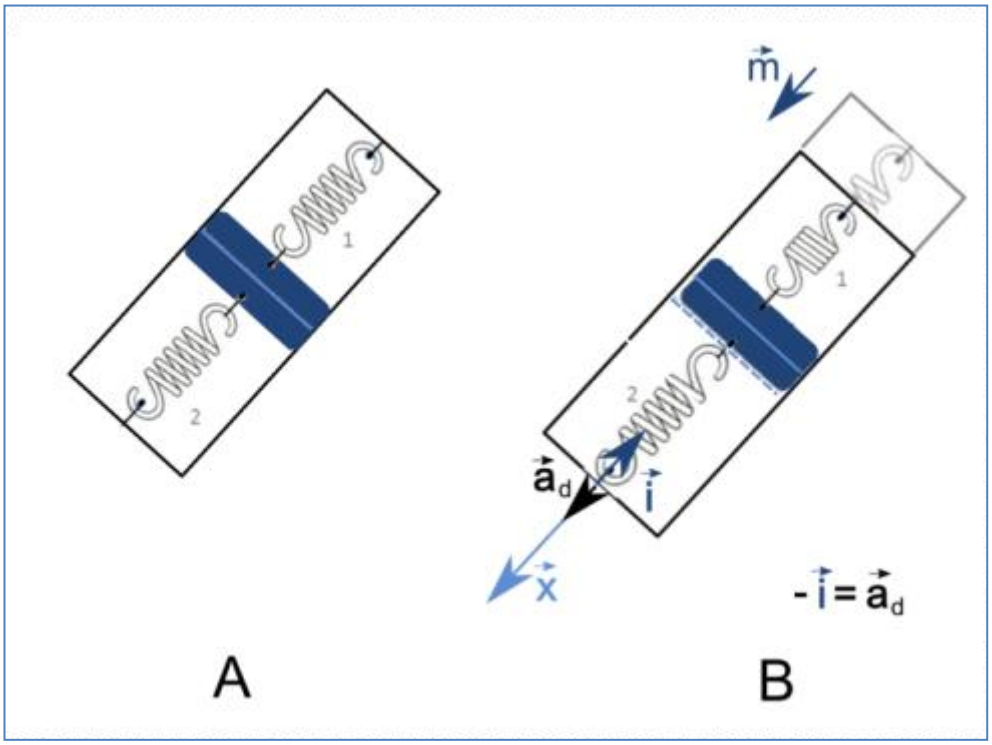


Figure 2.9 Representation of the effect of movement on an accelerometer. The accelerometer is schematically represented as a weight pulled by two springs, one on each side, able to move in only one axis in a cylinder (Axes called x in this illustration $\vec{x}$). Gravitational acceleration ($\vec{g}$) is not represented in this figure. **A.** The device is immobile but inclined to the right. **B.** The device is moved($\vec{m}$) in the direction of the x axis($\vec{x}$), this provokes inertia ($\vec{i}$). The accelerometer measures this inertia ($\vec{i}$) relative to the x axis($\vec{x}$), which is called the dynamic acceleration ($\vec{a_d}$). (Adaptation of Figure by Yves Handrich).

Amplitude and velocities of postural changes of an animal are typically relatively low relative to the dynamic acceleration experienced by the logger. This characteristic of body locomotion is used to separate the two components of measured raw acceleration. However, if the body orientation varies at a significantly slower rate than the instantaneous body

velocity, separation of both components (static and dynamic) is not possible (case not found in animals yet). To extract the static acceleration from the raw acceleration, a running mean (as in Wilson et al., 2006) or low-pass filter (fast Fourier Transform as in Sato et al., 2003, Watanabe et al., 2005 or adapted as in Fourati et al., 2009) can be applied on each axis, while dynamic acceleration is found by subtracting the static from the raw measures of acceleration (Wilson et al., 2006, Shepard et al., 2009). This project employed the low-pass filters from Fourati et al. (2009). Figure 2.10 illustrates an example analysis of a king penguin dive. This example has been chosen as the different components of acceleration are easier to visualise. Indeed change of posture during pedestrian gait is very small.

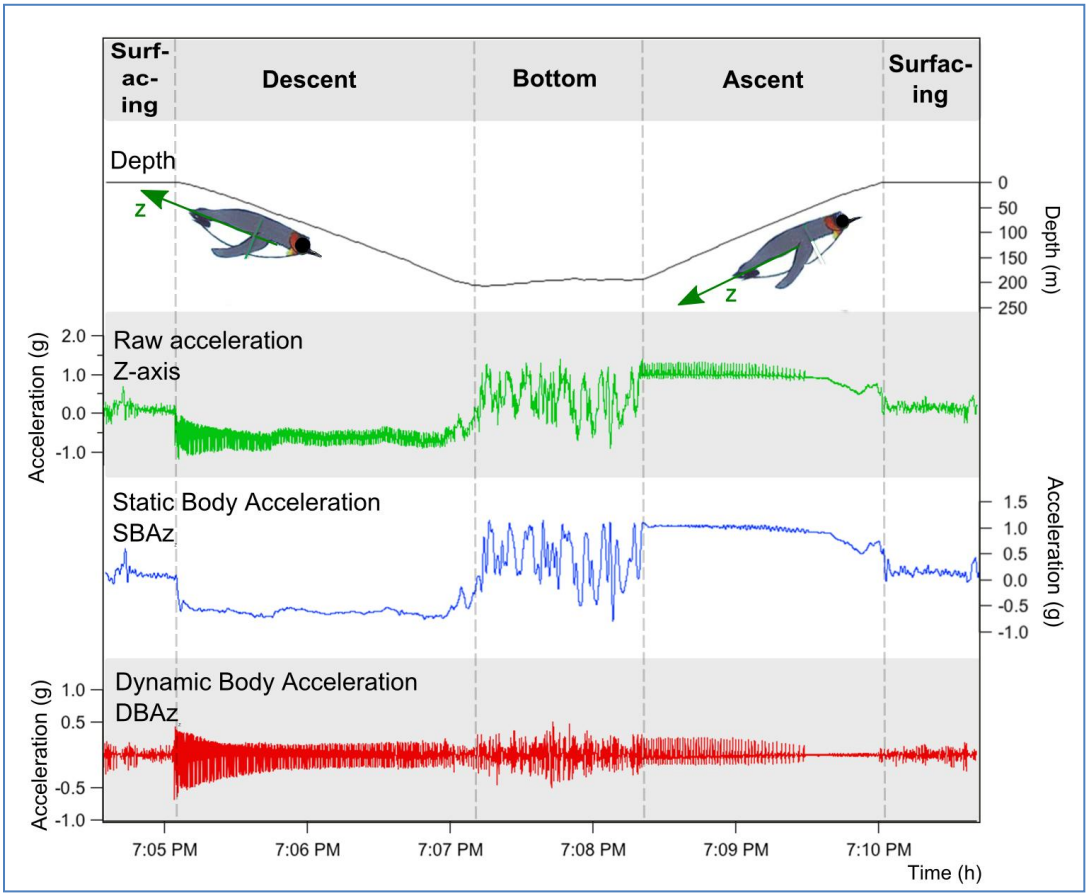


Figure 2.10 Graphical representation of the measured acceleration, and subsequent derivatives from it, in the z axis of an accelerometer instrumented to a king penguin during a dive at sea (Adapted from a Figure by Yves Handrich). In this example, water depth was also recorded (first line). The second line is raw acceleration measured in the z axis (Raw z-axis). The third line is the static body acceleration component of the raw data (SBA_z). The last line is the Dynamic body acceleration component (DBA_z) of the raw acceleration data.

Furthermore, to capture all important amplitude changes in accelerations experienced by the data logger, the recording frequency needs to be adjusted faster than the change of velocities related to the animal's movements (Gleiss et al., 2010). In this study, the frequency was 32.5 Hz.

2.1.7.1 Acceleration to estimate posture, angle and gait parameters

As the static component enables the observer to define the posture of the subject animal, the change in posture indicated by roll and pitch (Figure 2.11) can be defined. Yaw cannot be calculated as an additional triaxial magnetometer would have been required. Detailed anaylsis of dynamic body acceleration allows the identification of cyclical movements of gait such as steps and strides (Fourati et al., 2011) (in bipedial locomotion, one stride distance include two steps distance, Figure 2.15). Gait and posture were calculated from the accelerometry data using purpose-written software from the DEPE in Strasbourg, Logs (Yves Handrich, Strasbourg, France). The analyses were exported using custom-written computer programs by Yves Handrich in Matlab 6.0. (The MathWorks, Natick, MA, USA).

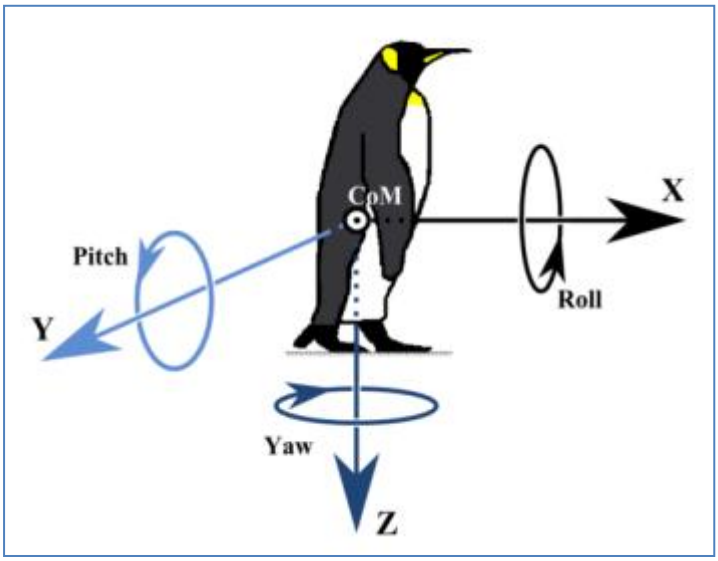


Figure 2.11 Illustration of the X-,Y- and Z- axes and their related angles changes relative to the centre of mass (hypothetical position), used in this thesis. Changes in measures of acceleration in the x-axis represent body roll, seen as leaning to the side. Change in the y-axis represents the pitch, seen as forward and backward lean. Yaw is represented as change in z-axis, but cannot be measured without an additional triaxial magnetometer.

2.1.7.2 Acceleration to estimate energy expenditure

The calculation of vectorial body acceleration (VeDBA) used in this project followed the protocol of Qasem et al. (2012) with the previously mentioned filter from Fourati et al (2009). The filter is a low pass filter, the fast Fourier Transform used in Watanabe et al. (2005) (§ 1.2.4) with adaptation for having a norm of the static body acceleration equivalent to 1 g. The analyses were performed using custom-written computer programs by Yves Handrich and adapted from Fourati (Fourati et al., 2009) in Matlab 6.0. (The MathWorks, Natick, MA, USA). All Statistical analysess and graphs were undertaken in R Cran (R Core Team, 2012).

2.1.7.3 ODBA versus VeDBA

A study which tested for a difference in the strength of the relationship between $\dot{V}_{O_2}$ and ODBA, and $\dot{V}_{O_2}$ and VeDBA, found a small but significantly stronger relationship between $\dot{V}_{O_2}$ and ODBA than between $\dot{V}_{O_2}$ and VeDBA (Qasem et al., 2012). However, the magnitude of ODBA is dependent on the orientation of the logger on the body (Qasem et al., 2012, Gleiss et al., 2010, Fourati et al., 2009), whereas since VeDBA corresponds to the mathematical equation of a sum of two vectors, it is independent of possible change of the logger orientation on the body. Thus in conditions where a constant orientation of the accelerometer cannot be assumed, using VeDBA is advised (Qasem et al., 2012). This suggests that when acceleration data are used to describe gait or overall body movements, when orientation is important, VeDBA may be more appropriate and consistent. Consequently VeDBA was the metric employed throughout this thesis to measure 3D movements or gait.

2.1.8 Kinematics

2.1.8.1 Materials

Due to the confined space, two cameras with wide angle lenses were required. The cameras were Prosilica GE680, 200Hz, from Allied Vision Technologies, Stadtroda, Germany. A NI

USB-6210 module, bus-powered M Series multifunction data acquisition (DAQ) module from National Instruments Corporation, Texas, USA was used to synchronise the cameras. The software used was Dynamic Vision Acquisition system (Alliance Vision, Montelimar, France). A black background was used to avoid any light reflections (directly into the camera, as well as via the translucent acrylic box).

2.1.8.2 Calibrations

A black and white draughtboard with boxes of 4*4 cm made with rigid plastic was used as a spatial reference.

Intrinsic parameters of the camera:

The first calibration allowed calculation of, and compensation for, the optic deformation from the camera (e.g. zoom). A video sequence was made in which the draughtboard was moved throughout the entire area where the bird could be, within the field of vision of each of the cameras. Digitisation of four external intersections of the draughtboard, via Loco 3.3 software (Loco 3.3. Paul-Antoine Libourel, Musée National d'Histoire Natuelle, Paris, France), enabled the software to calculate the deformation linked to the intrinsic parameters of the camera by re-establishing the real dimension of the spatial reference. This was automatically compensated for in the videos.

Extrinsic parameters of the both cameras:

A picture of the draughtboard in the field of vision of both cameras was used as the second calibration. This picture was critical as it enabled 3D reconstruction using the simultaneous videos of both cameras. Digitisation of the four external intersection of the draughtboard in Loco 3.3 software for the synchronised pictures of the both cameras enabled calculation of their exact spatial position. These extrinsic parameters were automatically taken into account by the software to enable calculation of the 3D position of the markers on the king penguin.

The camera setup was kept the same for the entire day, to minimise the need for repeat calibration. Thus, once the setup was finished and the associated calibration completed, every effort was made to avoid moving any of the cameras by touching them or stepping on cables, and to avoid any strong wind currents which could lead to the cameras vibrating. As a consequence, placing the subject penguin in the respirometery chamber was undertaken with great care (Figure 2.12).

Figure 2.12 Placement of a king penguin into the respirometer chamber. Top: The position of one of the calibrated cameras is highlighted by the circle in the confined experimental environment. King penguins need to be held horizontally and firmly when placed inside the respirometer chamber. **Bottom left:** The subject penguin is helped to stand. **Bottom middle:** the hood on the bird is removed. **Bottom right:** the chamber is replaced accurately to avoid leaks.

2.1.8.3 Bird preparation

To enable the 3D movements of the feet to be followed while the bird was walking, white out (Tipp-Ex®) was used to draw markers of approximately two cm diameter in the following locations of the foot, described for the left foot (Figure 2.13): **(A)** Marker placed on the left side of the ankle, close to the lowest point of the feathers. This marker was orientated relative to the current position of camera (frontward or backward, 2.1.8.4 and Figure 2.14 for more details about the cameras positions). **(B)** Marker placed on the left side of the ankle close to the angle formed by the ankle. This marker was again orientated relative to the current position of the camera (frontward or backward, 2.1.8.4 for more details about the cameras positions). **(C)** Marker was placed on the middle toe tarsus, at the limit formed with the claw. **(D)** Marker placed on the middle toe at the start of the metatarsus. The same markers were used for the right foot.

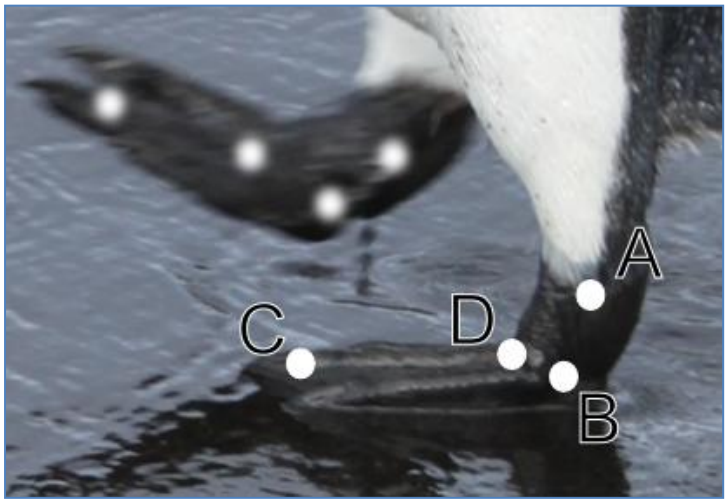


Figure 2.13 Position of the feet markers on the left feet. Anatomical positions A, B, C and D are explained in the text § 2.1.8.3.

2.1.8.4 Video collection protocol

Two synchronised videos were recorded at 50 Hz for 15 seconds while the penguin was walking. Due to the approximately cylindrical shape of the penguin leg, orientations of the camera view angles were less than 90°. This enabled the simultaneous view of, at least, two markers by both camera, such that a 3D view could be reconstructed (Figure 2.14). The 15-

second period included a minimum of 10 cycles of strides (of the same leg). Stride cycle is defined by the temporal interval from the initial contact of one foot to the next of the same foot. Step length is the distance of a step, while a step is defined as the toe-off (moment the entire foot has lost contact with the ground) to the initial contact of the same foot with the ground again. A stride is described by two phases: stance and swing phases (Figure 2.16), where the stance is limited to the time between the initial contact (first moment when the feet touch the ground) to the toe-off (moment that the toe leaves the ground), and the swing phase is the opposite. Duty factor is defined as the percentage of duration of the stance phase per stride duration (Abourachid et al., 2011).

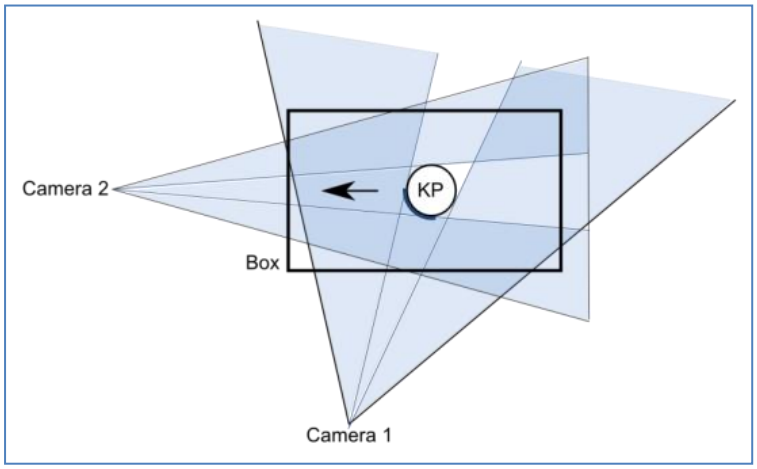


Figure 2.14 Overhead view from the frontward camera setup. The circle labelled KP represents the position of a king penguin while the arrow represents the direction of walking. The shaded area represents field recorded by the camera, and the dark areas on the king penguin represent the fields recorded by both cameras simultaneously where the markers were placed to enable a 3D vision.

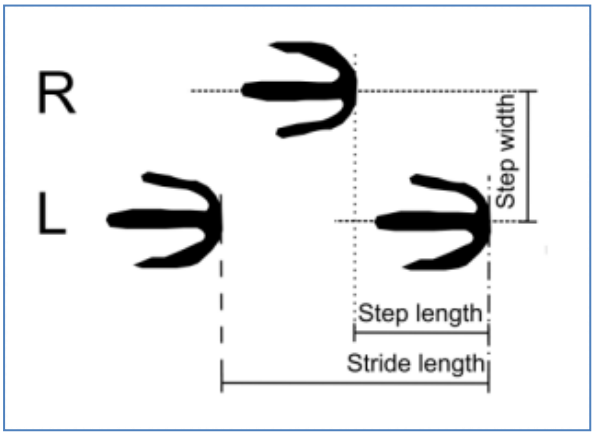


Figure 2.15 Schematic illustration of the step and stride distances of king penguin tracks. R is for right foot, L for the left foot.

The position of the cameras was changed each day. On the first day the camera was placed on the left side and in front of the penguin. On day two the cameras were placed on the left side and facing towards the dorsal side of the penguin. The data sets were obtained from pre-trigger recordings, whereby the 15 seconds previous to the recording activation were saved, which enabled 10 fluent walking cycles to be captured after their occurrence. The sequences were taken at speeds of 1, 1.2, 1.4 and 1.6 km/h, which was in the range of walking comfort of the king penguin, enabling a fluent walk. To enable a 3D reconstitution of the gait, at least two of the same marks needed to be visible to both cameras.

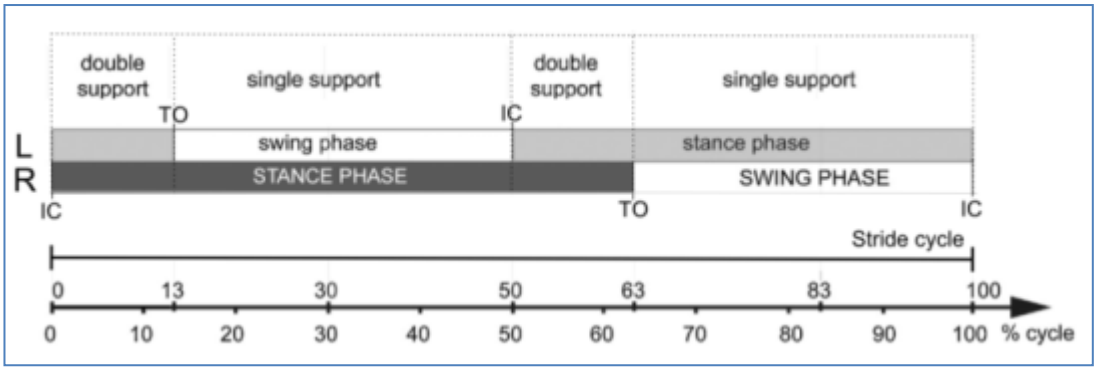


Figure 2.16 Example of a stride cycle of pedestrian locomotion and the definitions of walking phases. Time is expressed as percentage of the cycle duration. L represents the left foot and R the right foot. IC is for initial contact and TO for toe-off moment (from Abourachid et al., 2011).

2.1.8.5 Data collection and analysis

Unfortunately, due to malfunction of the Dyvas software at the start of the experiment during the second field season, the videos of the first recorded birds (subgroup of birds D) in their heavy state (day 0) could not be used. Thus to ensure a large change of mass across the experiments concerning birds of group D (at day 0 and day 22), data from the birds recorded later (second subgroup D), representing only four birds, were used. Only data from the left foot were used for gait analysis as it was found that the markers on the right foot were periodically hidden from the camera by the left foot. The step width was defined with data of both feet, calculated from using the initial contact position of the left foot and the following initial contact of the right foot, using the marker D (Figure 2.13). Movement of the treadmill was taken into account by adding the horizontal displacement due to the specific speed of

the belt. Each marker on the penguin was manually digitised, frame by frame with Loco 3.3. (Loco 3.3. Paul-Antoine Libourel, Musée National d'Histoire Natuelle, Paris, France). The task of accurately and precisely digitising was time-intensive so digitising was performed for one speed (1.4km/h) on the flat surface only, with birds of the second subgroup D (four birds). Due to problems with video or calibration quality, data were only available for four individuals at 'the heaviest', three at the 'heavy', two at the 'light' and four at the 'lightest' body mass conditions for digitisation (n=4, 3, 2, 4). 2D data positions were then translated into 3D coordinates using Loco 3.3. Spatial calculations via geometry as a function of time were performed in R Cran (R Core Team, 2012).

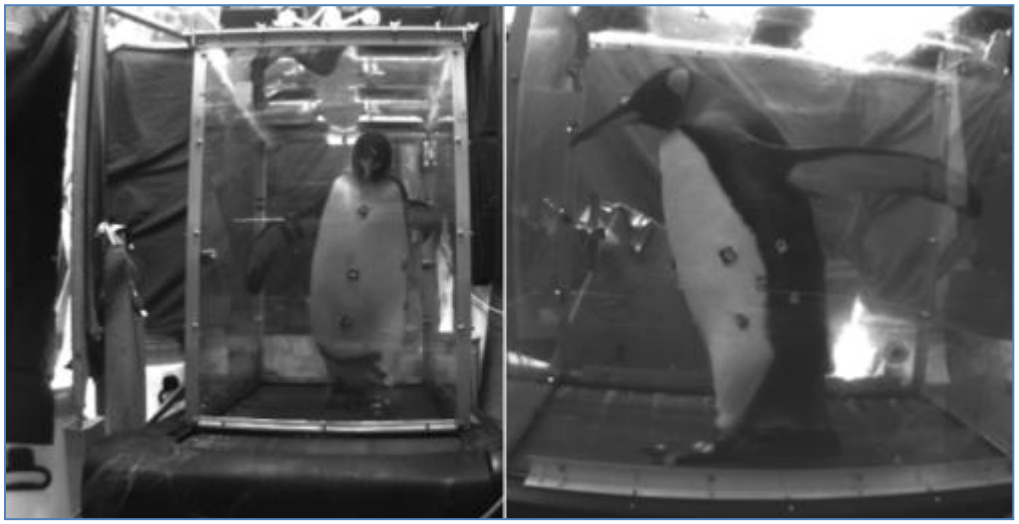


Figure 2.17 View of a penguin while walking from camera 1 (on the left) and from camera 2 (on the right).

2.2 Experimental protocols

2.2.1 Handling birds

King penguins were captured in La Baie du Marin colony. A hood was place over their head immediately upon capture to calm them. To minimise aggressive behaviour, the birds were touched as little as possible in all situations until their return to the colony. When it was necessary to handle the birds, they were touched on the thorax, close to the keel. This minimised their response. This technique was used during instrumentation of the loggers on the back of the bird (as in Figure 2.6), which may have helped to minimise their stress responses.

2.2.2 Experiment I: Stress response and acclimation

2.2.2.1 High activity birds

Later on the day of capture or on the following day, a bird in courtship (from group B) was taken from its pen, the bird was instrumented with the two data loggers (heart rate data logger § 2.1.6, and triaxial acceleration data logger § 2.1.7.). The bird was then placed in the respirometer chamber. The chamber was mounted upon a treadmill such that the birds walked at a controlled speed. $\dot{V}_{O_2}$ (§ 2.1.5.3), heart rate and VeDBA were measured, while the bird was subjected to two sets of three walking sessions at a speed of 1.4 km/h, each of 10 minutes duration and separated by 10 minutes rest (Figure 2.18 for an example of the experimental schedule). One set was performed with the presence of a stressor and the second set was performed in a quiet environment, i.e. while unstressed. The order of the sets was randomised. Before the unstressed set, the bird was allowed to rest for one hour, which was deemed sufficient time to remove any stress effects from previous experiments (Groscolas pers. obs.). The unstressed period consisted of leaving the bird alone in the respirometer while it walked for 10 minutes, without any additional noise aside from the noise of the colony. The stressed data are defined as the data collected during a session of 10 minutes with the presence of a stressor (§ 2.2.2.3) for the entire time. After the experiment the birds were released at the same place in the colony from where they had been caught.

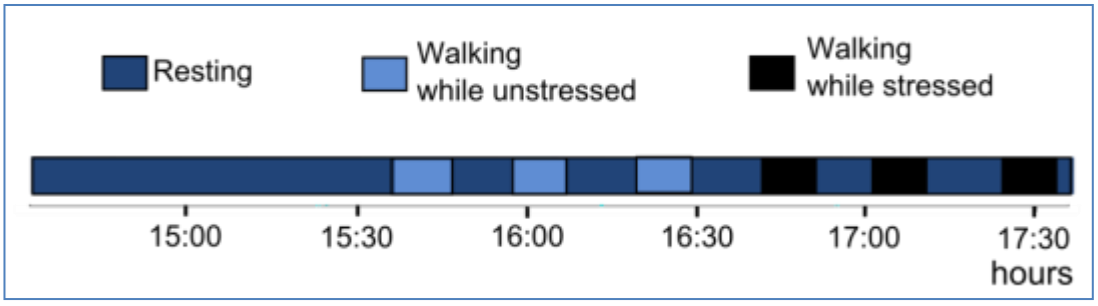


Figure 2.18 Example of a schedule of experiment I for bird at high activity (Table 2-1). The dark area indicates when the bird is in the respirometer chamber mounted on a treadmill. The light parts indicate when the bird is walking in an unstressed condition; the black parts indicate when the bird is walking in the stressing condition. The order of the unstressed/stressed sets were randomised however, the bird always rested for an hour before the unstressed set.

2.2.2.2 Low activity birds

Incubating birds were hooded to calm them, marked and then equipped *in situ* with the same data loggers as the birds tested in high activity (§ 2.2.2.1). These birds were the birds of group C (Table 2-1) The egg of the bird was simultaneously replaced by a plaster dummy egg and the real egg placed in an incubator at 37.5°C and 60% relative humidity. Subsequently, heart rate and accelerometry were recorded for a minimum of two hours while inside the colony (Figure 2.19 for an example of the experiment schedule). Then, the bird was transferred by hand, still in incubating posture with the dummy egg held against the brood patch, into a respirometer chamber located in a laboratory less than 20 m from the bird's nesting site. It was then left alone overnight for at least 10 hours, while $\dot{V}_{O_2}$, heart rate and VeDBA were monitored. The following day, the bird was submitted to four stressing periods of 15 minutes, with a resting period of one hour in between, while $\dot{V}_{O_2}$, heart rate and VeDBA were continuously measured. The stressed/unstressed conditions were similar to those of the walking birds, except the birds were incubating and therefore not expected to walk away from the 'egg'. The bird was subsequently transferred back to its original location in the colony, with its egg returned. It was observed for the following three days to ensure that it did not desert the egg.

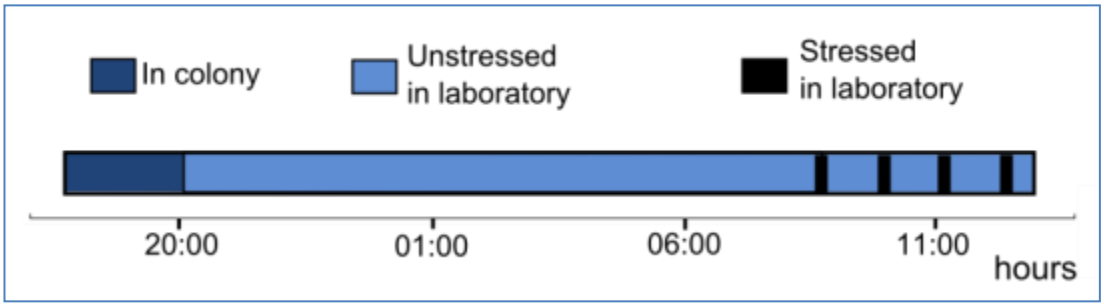


Figure 2.19 Example of a schedule of experiment I for a bird at low activity (Table 2-1). The dark area shows when the bird was monitored inside the colony. The light area indicates when the bird was in the respirometer chamber during the 'unstressed' condition, while the black represents the periods of the 'stressed' condition.

2.2.2.3 Stressor

Both visual and auditory stressors have been shown to impact on the behaviour and heart rate of king penguins (Viblanc et al., 2012a) and thus the stressor used in the present study included both types. The visual element was provided by the movement of the researcher (ASTW) while the auditory element consisted of noise generated by the striking together of two pieces of metal by the researcher. The researcher stood approximately two metres from the respirometry chamber at the start of the application of the stressor.

2.2.2.4 Stressed state

To avoid acclimation of the bird to the stressor, its intensity (distance to the animal, speed and intensity of the movement, amplitude of the sound and the frequency that the sound was generated) was adjusted. Initially, the effectiveness of increased heart rate as an indicator of a stressed state was (von Borell et al., 2007, Nimon et al., 1995, de Villiers et al., 2006, Ropert-Coudert et al., 2009, Culik and Wilson, 1991, Viblanc et al., 2012a) was pilot tested. Maintaining a constantly high heart rate in the subject bird was impossible without additional motions, and thus only the actions associated with vigilance behaviour were used as indicator of stressed state (Rushen, 2000). The objective was to achieve constant attention by the bird towards the researcher without an increase in motion (displacement behaviours excluded) throughout the period of the stressor. During the high activity condition, the same indicator was used while ensuring maintenance of an apparently fluid walk. As soon as a bird exhibited an increase in motion or an irregular walk, the stressor was decreased or momentarily stopped until the cessation of the additional motion or until fluid walking was restored.

2.2.3 Experiment II: Biomechanics and energy expenditure of walking king penguins

2.2.3.1 Walking birds

Penguins were captured in the morning and soon afterward their ability to walk on a treadmill was assessed. When enough penguins suitable for the treadmill had been captured,

data collection began. The first experiment generally took place on the same day or the day following capture. The penguins of group D were kept in a pen until the end of experiments (Table 2-1). Before the experiment, each bird was weighed and equipped in the same fashion as the birds used in § 2.2.2.1. The bird was then placed in the respirometer chamber upon a treadmill such that he walked at controlled speeds. The $\dot{V}_{O_2}$ and VeDBA of courting birds were measured as soon as the bird was put in the respirometer chamber. The bird rested for one hour in before the treadmill was turned one, thus requiring the bird to walk (Figure 2.20 for an example of the experiment schedule). Then, an initial walking session of five minutes was completed to acclimate the bird to walking on the treadmill. The experiment involved two sets of four walking sessions at speeds of 1, 1.2, 1.4 and 1.6 km/h, with 10 minutes rest between each. The speed order was randomised. One set of walking sessions was conducted on the flat, while another was conducted on a 13% incline; the order of the two sets were randomised. Experiments and data collection were repeated four times at approximately days 0, 7, 14 and 21, with the respective average body masses referred to as 'heaviest' (13.2 kg), 'heavy'(11.7 kg), 'light'(11.0 kg) and 'lightest' (9.8 kg). Birds were kept in a pen after the experiment and released at the same place in the colony after the fourth experiment.

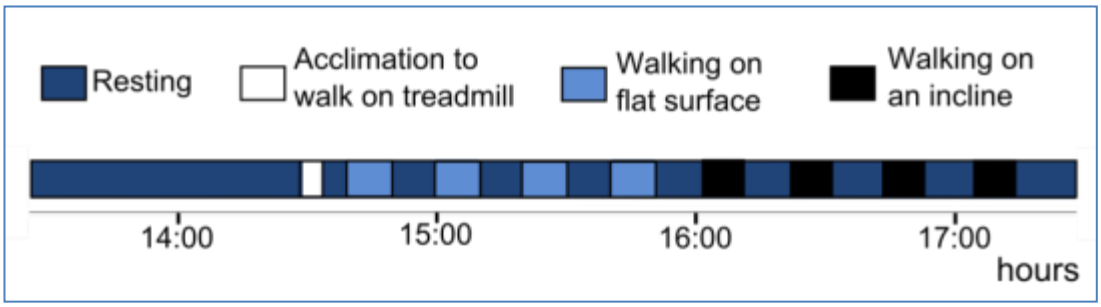


Figure 2.20 Example of a schedule for experiment II (Table 2-1). The dark area is when the bird is resting in the respirometer chamber. The white represents the first walking session, used as an acclimation to walking on a treadmill. The light parts indicate when the bird walked on the flat, while the black parts show when the bird walked on an incline. The order of both sets of four walking session were randomised. One set involved four walking sessions at the randomised speeds of 1, 1.2, 1.4 and 1.6 km/h.

2.2.3.2 Location of the centre of mass

The location of the centre of mass was determined using the multiple suspension method (Abourachid, 1993), on the body of both acquired king penguin cadavers (individuals of groups E, Table 2-1). In a rigid body, the centre of mass can be determined by suspension (with a rope, for example) of the body from different places on the body. The rope axis will always intersect the centre of mass of a stabilised, suspended body that is free of movement. Photoshop (Adobe Elements 6.0) or Inkscape (Inkscape 0.48, www.inkscape.org) software were used to visually determine the centre of mass from photography of cadaver suspension.

2.3 Data analyses

2.3.1 Synchronisation of different physiological and biomechanical measures.

Simultaneous analysis of different measures (e.g. physiology, biomechanics) was fundamental to achieve the objectives of this thesis. For example, to understand the paradox of optimised cost of walking fat penguins (chapter five, bird group D), simultaneously collected data on estimates of energy expenditure (respirometry) and biomechanics (accelerometry and videos) were used, while to define the cardio-respiratory stress response *per se* of the bird (chapter three, birds group B and C), simultaneously collected data from respirometry, heart rate and accelerometery were used. However each measure can include a different time lag due to both variations in equipment time lag and in rates of the physiological responses being measured. To synchronise these different measures, these varying time lags were accounted for. Explanations for controlling the lag due to the equipment have been discussed in specific paragraphs (as in § 2.1.5.3 concerning the respirometer). To use the 'instantaneous equation' to calculate $\dot{V}_{O_2}$, a good ventilation is essential. However the position of the bird within the chamber cannot be controlled. Thus, the distance of the bird to the location in the respirometer that the excurrent air leaves may vary (it is especially true for walking birds), which may lead to fluctuations of the gas concentration as this air may not be properly mixed, leading to potential error calculating the

instantaneous $\dot{V}_{O_2}$. For this reason, the minimal interval of data used for a mean was defined as an interval of four minutes (as in chapter three). All other techniques recorded the reaction of the birds' physiology or biomechanics almost instantaneously. Physiological time reactions differ depending on the kind of physiological response (e.g. stress response, activity), thus the different responses were tested to enable comparison of simultaneous data.

2.3.1.1 Reaction to activity

When an animal increases its activity level (e.g. starts walking), the increase in movement is instantaneously measured by the accelerometer. However a lag exists between the ATP demand from the muscle cells and its supply. In most species, the first source of ATP comes from phosophocreatine (PCr). Studies on nuclear magnetic resonance spectroscopy have shown the presence in equilibrium of PCr, ATP and ADP (Biewener, 2003) (Equation 2-3), especially in skeletal (and cardiac) cells, enabling a fast response to the myofibril ATP demand.

Equation 2-3

$$PCr + ADP \rightleftharpoons Cr + ATP$$

Simultaneously, the glycolysis (anaerobic) and oxidative respiration (aerobic) pathway to generate ATP are activated, which represents a delay in reaction time (Figure 2.21). Finally, when the ATP demand can be supplied aerobically, glycolysis is shut down. The accumulated deficit in PCr, as well as the lactate accumulation from glycolysis are recovered after the cessation of effort (Biewener, 2003). Oxygen delivery to the mitochondria is a cascade of processes with an important role for heart rate. Indeed blood is the vessel transporting oxygen to the cell, thus the reaction time of oxidative respiration depends on heart rate reaction time. Heart rate is initiated and set by the sinoatrial node and atrioventricular node, which are regulated by the sympathetic and parasympathetic nervous systems. Heart rate has been shown to react within less than one minute (von Borell et al., 2007). Piloted walking sessions indicated that a 10-minute duration resulted in stabilised

measures of $\dot{V}_{O_2}$, heart rate and body movement, confirming previous studies (Halsey et al., 2007b, Fahlman et al., 2004). Graphical representations of the responses of the three aforementioned physiological parameters' reaction time as a function of time can be seen in Figure 2.22.

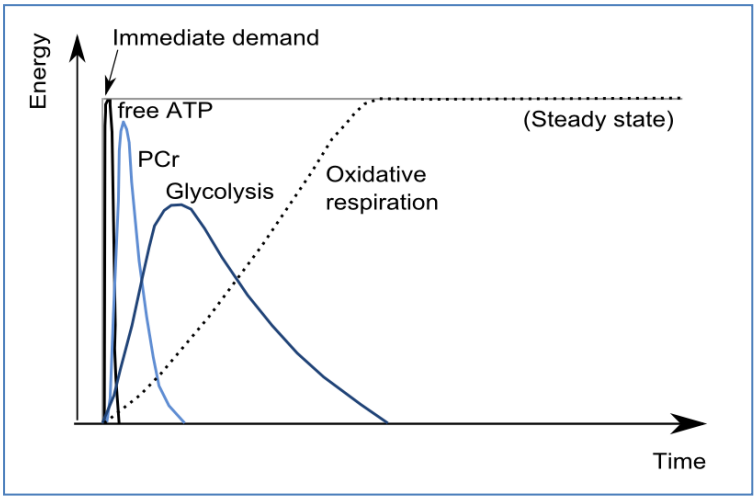


Figure 2.21 Source of ATP as a function of the time. Diagram showing how the immediate demand for an increase in ATP supply (grey line) to the muscle of an exercising animal is met by various sources of metabolism during the start of exercise. PCr is for phosphocreatine, and ATP for adenosine-5'-triphosphate. From Biewener (2003)

Visual inspection of $\dot{V}_{O_2}$ plotted against time enabled an approximation of the time lag of this measure to be defined. The lag was estimated to be one minute. Graphical comparison of individual- and average $\dot{V}_{O_2}$ data, subsequent to the first minute being removed, was a good approximation of the stabilised (i.e. plateau) rate of oxygen consumption calculated without instantaneous transformation ($\dot{V}_{O_{2w}}$). To take into account possible change of the respiratory quotient ($\frac{\dot{V}_{CO_2}}{\dot{V}_{O_2}}$), equation 11.7 in Lighton (Lighton, 2008) was used:

Equation 2-4

$$\dot{V}_{O_{2w}} = \frac{\dot{V}_c * \left(F_{i_{O_2}} - F'_{e_{O_2}}\right) - F_{i_{O_2}} * \left(F'_{e_{CO_2}} - F_{i_{CO_2}}\right)}{1 - F_{i_{O_2}}}$$

; where $F_{i_{O_2}}$ is the fractional O_2 concentration in the air entering the chamber (incurrent); $F_{i_{CO_2}}$ is the fractional incurrent CO_2 concentration; $F'_{e_{O_2}}$ is the fractional excurrent O_2

concentration, with water removed (denoted by the '); $F'_{e_{CO_2}}$ is the fractional excurrent CO_2 concentration, with water removed; and $\dot{V}_c$ is the corrected mass flow rate for the water vapour pressure, using the equation 8.6 in Lighton (Lighton, 2008), as water was removed before measuring the percentage of the different gases.

Equation 2-5

$$\dot{V}_c = \frac{\dot{V} * (BP - WVP)}{BP}$$

, where $\dot{V}$ is the uncorrected flow rate, BP is the barometric pressure; WVP is the water vapour pressure. Consequently, the mean data from the 10-minute walking sessions were calculated from the entire 10-minutes data for heart rate and accelerometry, while $\dot{V}_{O_2}$ was calculated with the first minute removed.

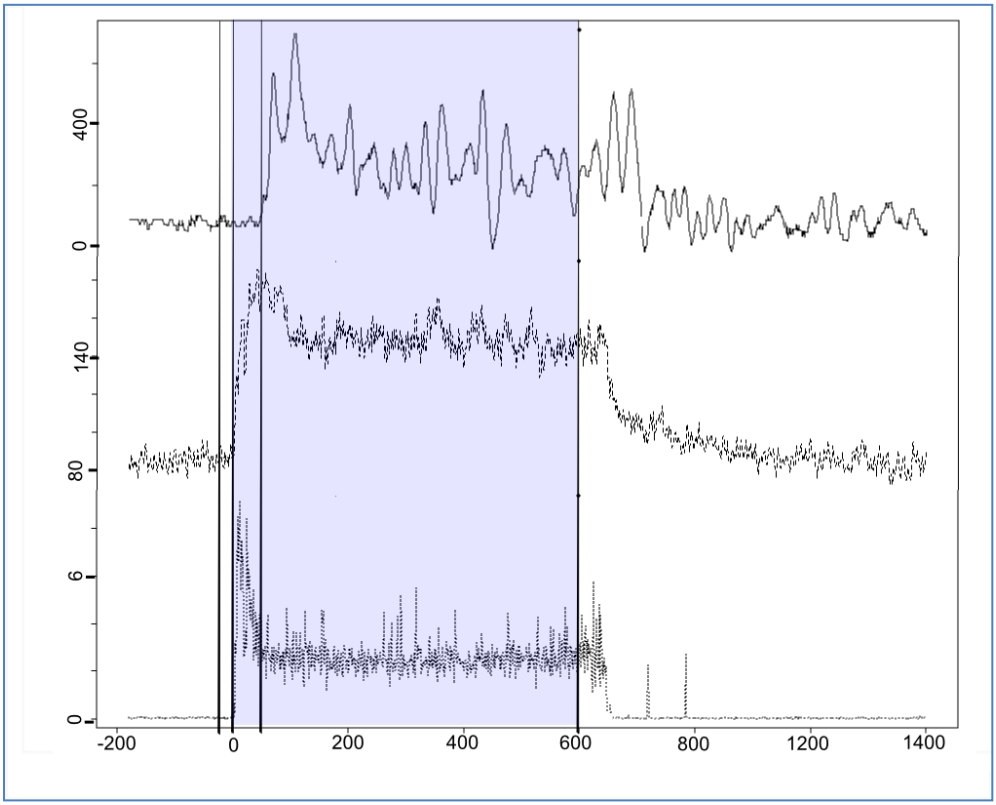


Figure 2.22: Graphical representation of $\dot{V}_{O_2}$, heart rate and VeDBA of a king penguin during a walking session as a function of time (sec). t=0 is the start of the walking session, and t=600 its end. The shaded area represents the entire walking session. The first vertical line represents 30 sec before the walking session. The second to the third vertical line represents the first minute of the walking session, which was disregarded when calculating mean $\dot{V}_{O_2}$ for the walking session. To improve visual representation, a 10-second running mean was calculated for $\dot{V}_{O_2}$.

2.3.1.2 Reaction to a stressor

To define the reaction time to a stressor of each physiological parameter, the same graphical analysis was undertaken as when the response to an activity was defined (§ 2.3.1.1). This resulted in the same observations: heart rate and accelerometry reacted almost instantaneously while $\dot{V}_{O_2}$ needed approximately one minute to react (Figure 2.23). A duration of 15-minutes for the stressing session was defined, to ensure a stabilisation of the stress response. Consequently mean data for a stressing session were calculated from 15-minutes data for heart rate and accelerometry, while the first minute was removed for the $\dot{V}_{O_2}$ mean.

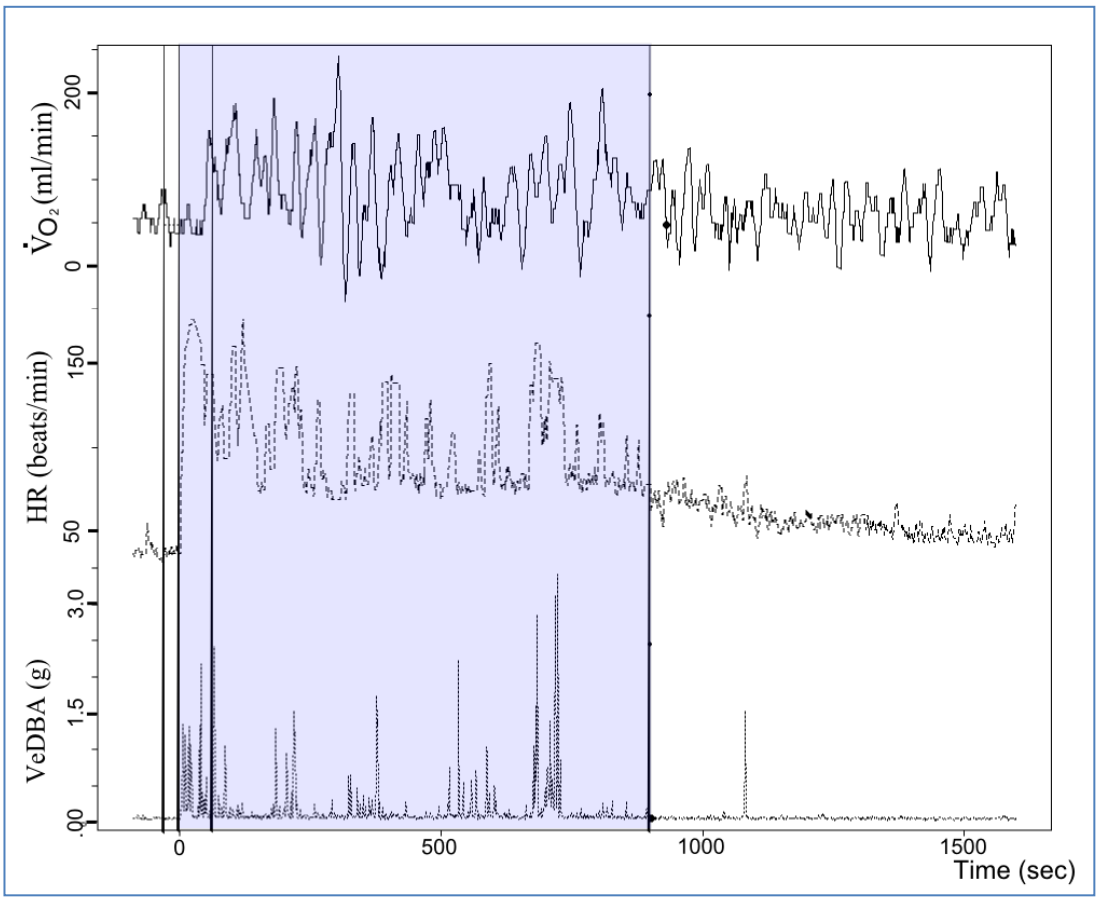


Figure 2.23: Graphical representation of $\dot{V}_{O_2}$, heart rate and VeDBA of a bird at low activity as a function of the elapsed time (sec). t=0 is the start of the stressor. The shaded area represents when the stressor is present (end at t= 900). The first vertical line represents 30 sec before the start of the stressor, while the third vertical line is placed at t=1 min after the start of the walking session. To improve the visual representation, a 10-second running mean was calculated for the $\dot{V}_{O_2}$ data.

2.4 General statistical analysis

Due to the amount of data and the different outputs from each of the software types, R Cran software (R Core Team, 2012) was used for data importation and analyses. Some analyses were double-checked in SPSS. The bioportal website (https://www.bioportal.uio.no/) was used for particularly large data files. Scripts were also made in Microsoft Access to 'cross reference' the data. Unless otherwise specified, all computational transformations and analyses of data were undertaken using R 2.13.1 software, R Cran (R Core Team, 2012). Inkscape software© was used to customise the graphs. Specific Statistical analyses are explained in each results chapter.

3. Reassessment of the cardio-respiratory stress response: accounting for movement.

This chapter studied the stress response of king penguin to enable description of the changes happening in their cardio-respiratory and behavioural systems, as well as the cost of these changes. However, one factor appeared to be important to quantify and compensate for: the movement induced by the stressor. Indeed, the proportion of change in the cardio-respiratory system is partly due to increases in activity level (i.e. behavioural stress response: 'fight or flight' response). This issue is discussed in this chapter.

3.1 Abstract

The typical cardio-respiratory stress response involves an increase in heart rate, as well as an increase in $\dot{V}_{O_2}$. Previous research has shown that the increase in heart rate is higher than the increase in $\dot{V}_{O_2}$, calling this phenomenon 'additional heart rate'. However, little is actually known about the importance of the effect of movement on the cardio-respiratory stress response. No studies have looked at the cardio-respiratory stress response during high activity. Moreover the 'overall stress response' also includes the behavioural 'fight or flight' response, which can cause an increase in striated muscle activity (called change of 'motion' in the present study) between unstressed and stressed conditions. Attempts to minimise these changes in motion have been made (e.g. by protocol, which needs contribution of conscious subjects) to measure the 'stress response *per se*'. However, none of the previous procedures attested for the same motion levels between both stress conditions. Moreover, displacement behaviours (e.g. scratching) have been shown to help coping with a stressor, and prohibiting the subject to move freely may bias the stress response itself. Consequently, the cardio-respiratory 'stress response *per se*' (compared to the 'overall stress response') is still unknown. Therefore, this study measured the cardio-respiratory (via heart rate and the $\dot{V}_{O_2}$, respectively) and motion levels (via vectorial dynamic body acceleration VeDBA) of king penguins (*Aptenodytes patagonicus*), during experimental conditions of 10 to 15 minutes defined as 'stressful' and 'not stressful', during both low and high activities. Findings were that (1) at high activity condition, overall stress response is only an increase of mean $\dot{V}_{O_2}$. (2) Same results were found for the stress response *per se*, as the high activity prevent additional motion or heart rate. While at low activity, (3) the overall stress response was an increase in mean $\dot{V}_{O_2}$, heart rate and VeDBA, which is in accordance with previous research. However (4), the stress response *per se* (i.e. controlling for levels of motion) during low activity, only mean $\dot{V}_{O_2}$ changed in response to a stressor, contradicting the popular idea of the stress response being mainly an increase in heart rate. These results highlight the importance of

including movements, i.e. the initial activity and change in motion, when measuring the cardio-respiratory stress response.

3.2 Introduction

Laboratory experiments that tested the cardio-respiratory response to psychological stressors have usually recorded mean heart rate and $\dot{V}_{O_2}$ during low activity such as piloting, avoiding shocks. The repeated findings across the few species (humans, rats, dogs) and psychological stressor types that have been studied are that the cardio-respiratory stress response is described by an increase in both $\dot{V}_{O_2}$ and heart rate, with heart rate increasing to a value greater than expected given the increase in $\dot{V}_{O_2}$ (Blix et al., 1974, Carroll et al., 1986, Boerth et al., 1969, Langer et al., 1979). This greater increase in heart rate has been quantified by comparing the recorded value of heart rate during the presence of the stressor with heart rate predicted for the concomitantly recorded $\dot{V}_{O_2}$, using calibration curves derived from graded exercise (Figure 3.1). The resultant high heart rate relative to $\dot{V}_{O_2}$ has been termed 'additional heart rate' (Blix et al., 1974, Stromme and Ingjer, 1978) or 'additional cardiac output' (Carroll et al., 1991, Sherwood et al., 1986).

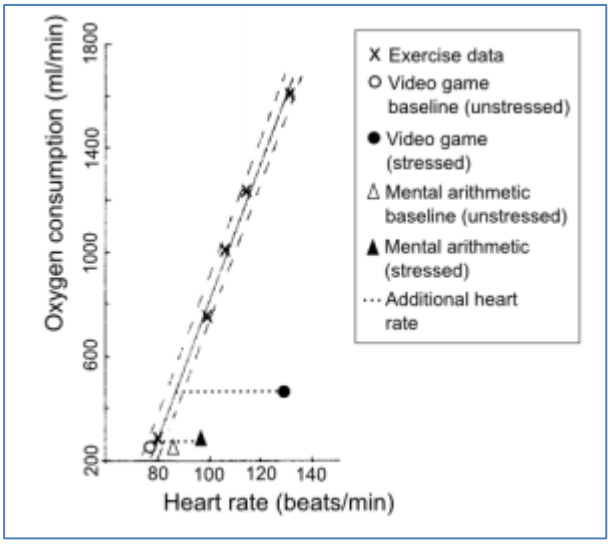


Figure 3.1 Visualisation of the additional heart rate. Graphical representation of oxygen consumption as a function of heart rate for one individual. Modified from Turner and Carroll (1985). The calibration data collected during exercise are symbolised by crosses. Baseline data were collected while resting (plain circle and triangle). The stressed data are in black (circle: while playing video game, and a triangle symbolises the data collected while doing mental arithmetic.). The additional heart rate (Δx) related to the calibration found during exercise (plain line) are represented with the dotted lines.

Heart rate is the most typical measure used to assess the response to a short term psychological stressor (i.e. from fear, as opposed to a physical stressor such as thermic or chemical stressors) in animals and humans. Changes in heart rate occur almost immediately, are relatively easy to record, and typically represent a more sensitive response than outward signs of stressed state, such as changes in behaviour (Nimon et al., 1995, von Borell et al., 2007). Thus, this metric has been applied to a wide range of animals including farmed breeds of cows (*Bos taurus*), pigs (*Sus scrofa domesticus*), horses (*Equus caballus*) and goats (*Capra hircus*; von Borell et al. 2007), albatrosses (*Diomedea exulans;* Weimerskirch et al., 2002), giant petrels (*Macronectes halli;* de Villiers et al., 2006), koalas (*Phascolarctos cinereus;* Ropert-Coudert et al., 2009) and several species of penguin (Adelie *Pygoscelis adeliae*, Culik and Wilson, 1991; gentoo *Pygoscelis papua*, Nimon et al., 1995; king *Aptenodytes patagonicus*, Viblanc et al., 2012a).

To date there are no studies measuring the cardio-respiratory stress response at high activity. A few studies have tested the $\dot{V}_{O_2}$ of swimming fish after different stressors (Davis and Schreck, 1997, Barton and Schreck, 1987). For instance, Barton and Schreck (1987) measured the $\dot{V}_{O_2}$ in juvenile rainbow trout (*Oncorhynchus mykiss*) while highly active (i.e. swimming against a fixed artificial current), for a group that had been exposed to an acute stressor (being handled out of the water) and for a group that had not been exposed to a stressor. $\dot{V}_{O_2}$ was approximately double in the stressed fish, demonstrating the cost of stress response on a short-term scale. However, heart rate was not measured in this study and a constant flow rate of water by no means rules out some variation in motion levels between the stressed and unstressed conditions.

The ‘disturbance’ stress response can include behavioural modifications epitomised by the concept that the response evokes preparation for “fight or flight” response (Cannon, 1929) or

the updated "freeze, flight, fight or fright" response (Bracha et al., 2004). However, small changes in motion levels such as mastication can cause relatively large increases in heart rate (Major, 1998). None of the aforementioned studies investigating the cardiac or cardio-respiratory response to a disturbance stressor measured the proportion of modification in the response due to changes in motion levels between stressed and unstressed conditions of low activity. Yet in the majority of those studies, movements were an inevitable element of the stressor response (e.g. piloting a plane during landing or taking off, Blix et al., 1974, or to avoid a shock, Langer et al., 1979), and thus measures of $\dot{V}_{O_2}$ and heart rate represent not only the cardio-respiratory response to stress '*per se*' (i.e. changes in physiological activities as increases in hormones, up-regulation of the cardio-respiratory system, Moberg and Mench, 2000, Brener, 1987) but also the response to small increases in physical activities levels from striated muscle (called 'motion' in this study), i.e. the 'overall stress response'. Therefore, in cases where motion levels increase in response to a stressor, even if this is only slight and thus close to imperceptible, elevations in $\dot{V}_{O_2}$ and heart rate will be due to both a stress response *per se* and a change in behaviour (von Borell et al., 2007).

Some studies on humans have employed protocols that aimed to minimise movements in the participants, and thus likely served as a partial control for the potential confounding factor of changing motion levels. For example, in a study by Turner and Carroll (1985) the stressor was answering mental arithmetic questions to compete for a prize and only the index finger should be raised (Turner and Carroll, 1985). In animals, to minimise the change of motion level, Von Borell advises measuring the effects of a stressor only during periods when the behaviour exhibited was similar to that when measurements were taken without the presence of a stressor (von Borell et al., 2007). Yet in all these cases motion levels are only approximately controlled for. The only method used to date to control for motion during stressing experiments is paralysis. Several stress studies have measured the heart rate of

fully paralysed by curare rats (e.g., Hahn and Slaughte.J, 1970, Trowill, 1967) and dogs (Church et al., 1966), which therefore fully removes the confounding factor of motion. Curarised rats and dogs showed an increase in heart rate during exposure to a shock; however curare itself causes a confound as it has also been reported to affect heart rate (Hahn, 1970). Furthermore, there is evidence that, in humans at least, displacement behaviour such as scratching can help to cope with the stressor and thus any methods that restrict such behaviour may hinder the natural stress response (Mohiyeddini and Semple, 2013, Wechsler, 1995, Maestripieri et al., 1992).

Thus, the *per se* effects of psychological stressors on the cardio-respiratory system are not clear, particularly in unrestrained subjects. Additionally, very little is known about the stress response during high activity. This knowledge would help providing a better understanding of the cardio-respiratory stress response. This would be especially useful to optimise and widen the use of heart rate as an index of stress state while an animal is active, as for research measuring the wellbeing of animals where movement may bias the results. Additionally, knowing the increase in $\dot{V}_{O_2}$ will give an initial idea of the short-term cost of the overall and *per se* stress response. Finally, defining the cardio-respiratory stress response of king penguins will enable testing of their acclimation to experimental environments (chapter four). Indeed, this will enable the removal of any potential confound while measuring the energy expenditure of king penguins in different situations. To have a better understanding of the importance of movement on the cardio-respiratory stress response, the present study compared $\dot{V}_{O_2}$, heart rate and motion across two activity levels in fully mobile animals, using king penguins as a study species. King penguins are especially suitable for such a study as two naturally occurring periods of low activity and high activity have been observed. First, while a king penguin incubates an egg, it remains sedentary and lacks motivation to flee in stressful situations, such as the presence of predators, which will result

in reflecting stress responses at low activity. In this study, wild incubating birds were temporarily given a dummy egg to incubate while resting within a respirometer and then exposed to a psychological stressor. Second, during the period of courtship, king penguins are active, walking within and around their colony. Birds in this phase of the breeding cycle were placed on a treadmill at a constant speed within a respirometer and intermittently exposed to the same stressor as the incubating birds. While incubating individuals are expected to remain sedentary (i.e. low activity) and individuals in courtship are expected to continue walking (i.e. high activity) during exposure to a stressor, in both cases this does not rule out the possibility of minor behavioural changes in response to the stressor. Additionally, the birds were instrumented with a miniature acceleration data logger, which is highly sensitive to changes in animal posture and motion levels (Yoda et al., 2001, Wilson et al., 2006, Fourati et al., 2009), enabling motion to be controlled for without inhibiting the natural responses of the animals to the stressor, as well as measuring the stress response at two different activity levels. Therefore, the aims of this experiment were to determine the importance of movements in the cardiac and respiratory stress responses of king penguins, (1) by looking at the effect of the initial activity on the cardio-respiratory stress response, and (2) by differentiation of the 'overall' and the '*per se*' stress responses in both of the initial activities presented, i.e. using VeDBA as a control for similar motion for the description of the stress response *per se*. It was hypothesised (1) that birds at high activity would increase $\dot{V}_{O_2}$ only for their overall cardio-respiratory stress response, as they will already have a high heart rate and VeDBA. (2) Their stress response *per se* would be similar to overall stress response, as no additional motion will be measured in VeDBA, due to the initial high activity level. Regarding a stressing condition at low activity (3), birds would exhibit the typical increase in the cardio-respiratory system (i.e. Heart rate and $\dot{V}_{O_2}$) as well as an increase in VeDBA for an overall stress response, while (4) the stress response *per se*

would only be an increase in $\dot{V}_{O_2}$, as no increase of heart rate would be needed as motion is similar.

3.3 Materials and methods

3.3.1 Birds and experimental protocol

Stress responses of high activity birds:

A bird in courtship (from group B; Table 2-1) was taken from its pen, later on the day of capture or on the following day. The bird was instrumented with the two data loggers (heart rate data logger § 2.1.6, and triaxial acceleration data logger § 2.1.7.) and then placed in the respirometer chamber. The chamber was mounted upon a treadmill such that the birds walked at a controlled speed. $\dot{V}_{O_2}$ (§ 2.1.5.3), heart rate and VeDBA were measured, while the bird was subjected to two sets of three walking sessions at a speed of 1.4 km/h, each of 10 minutes duration and separated by 10 minutes rest (Figure 2.18 for an example of the experimental schedule). One set was performed with the presence of a stressor and the second set was performed in a quiet environment, i.e. while unstressed. The order of the sets was randomised. Before the unstressed set, the bird was allowed to rest for one hour, which was deemed sufficient time to remove any stress effects from previous experiments (Groscolas pers. obs.). The unstressed period consisted of leaving the bird alone in the respirometer while it walked for 10 minutes, without any additional noise aside from the noise of the colony. The stressed data are defined as the data collected during a session of 10 minutes with the presence of a stressor (§ 2.2.2.3) for the entire time. After the experiment the birds were released at the same place in the colony from where they had been caught.

Stress responses of low activity birds:

Incubating birds were hooded to calm them, marked and then equipped *in situ* with the same data loggers as the birds tested at high activity (§ 2.2.2.1). These birds were the birds of group C (Table 2-1) The egg of the bird was simultaneously replaced by a plaster dummy

egg and the real egg placed in an incubator at 37.5°C and 60% relative humidity. Then, the bird was transferred by hand, still in incubating posture with the dummy egg held against the brood patch, into a respirometer chamber located in a laboratory less than 20 m from the bird's nesting site. It was then left alone overnight for at least 10 hours, while $\dot{V}_{O_2}$, heart rate and VeDBA were monitored. The following day, the bird was submitted to a stressor for one period of 15 minutes, while $\dot{V}_{O_2}$, heart rate and VeDBA were continuously measured. The stressed/unstressed conditions were similar to those of the walking birds, except the birds were incubating and therefore not expected to walk away from the 'egg'. The bird was subsequently transferred back to its original location in the colony, with its egg returned. It was observed for the following three days to ensure that it did not desert the egg.

3.3.2 Data Processing and Statistical analysis

Stress responses of high activity birds:

Regarding the stress response at high activity, the means of $\dot{V}_{O_2}$, heart rate and VeDBA per walking session (i.e. six walking session in total per bird) were calculated. As discussed in the General Methods chapter (§ 2.3.1.2), $\dot{V}_{O_2}$ of king penguins needed almost one minute to react. Thus, $\dot{V}_{O_2}$ was calculated as the mean value for the entire walking session excluding the first minute. Finally, grand means of $\dot{V}_{O_2}$, heart rate and VeDBA were calculated by means of the two last walking sessions, of each set of unstressed or stressed conditions; this was to avoid the potential simultaneous and additional stressor of being the first walking session. Indeed the first walking session while stressed was also removed as multiple stressors have been shown to modify the stress response (Dallman et al., 1992). These two grand means were used to represent the unstressed or stressed data of highly active birds. The grand means of VeDBA represented the amount of motion level. VeDBA was recorded while the birds were unstressed and while stressed. Values were compared using Wilcoxon signed-ranks tests and showed no significant statistical difference ($P = 0.23$), meaning that at high activity the motion levels were similar regardless of stress levels. Thus there was no

need to select periods where body motion levels were similar while the birds were stressed and unstressed to obtain the stress response *per se*. Indeed, in this case, comparison of unstressed and stressed parameters represented both the overall stress response (i.e. any changes due to the presence of a stressor including potential change in motion) and the stress response *per se* (i.e. changes due solely to the physiological stress response and not due to increased body motion associated with the stressor.). The populations from which the data were sampled were normally distributed as tested by the Shapiro-Wilk test. As population number was low, non parametric tests as Wilcoxon signed-ranks tests were done. However as the precision of the test for this sample number is only about ±0.025, t-tests were done simultaneously. T-tests results were always looked at to see the tendency and had priority when the p-value of the Wilcoxon signed-ranks tests was in the range of 0.05 ±0.025. This chapter only show p-value from the t-test to avoid confusion (This reasoning has been applied throughout the thesis). Therefore paired tests were conducted to test the difference between unstressed/stressed data, with similar motion between both conditions; N = 6 in both cases. See Table 3-1 for a summary of all analyses.

Stress responses of low activity birds:

Mean $\dot{V}_{O_2}$, heart rate and VeDBA of the birds in the low activity condition for the five minutes of lowest mean $\dot{V}_{O_2}$ during the unstressed period were calculated. The unstressed periods were considered as the hours of rest within the laboratory prior to the experiments (i.e. 10-14 hours) but during the daytime to standardise for the effects of circadian rhythms on metabolic rate (Halsey et al., 2008b), since experiments were undertaken during daylight hours. As king penguins are observed to recover from handling in approximately one hour (Groscolas' personal observations of heart rate data, and standard used protocol from previous studies measuring $\dot{V}_{O_2}$ of penguins; Halsey et al., 2007b, Green, 2001, Fahlman et al., 2004), the first hour of data after the handling was not included in analysis. These values

were taken to represent $\dot{V}_{O_2}$, heart rate and VeDBA of unstressed birds at low activity. The means of $\dot{V}_{O_2}$, heart rate and VeDBA, representing the data of stressed birds at low activity, were calculated for the incubating birds during stressing sessions, following the same protocol as birds of high activity. Wilcoxon signed-ranks tests showed that the means of VeDBA (representing motion levels) of the incubating birds during the stressed condition were significantly higher than during the unstressed condition (P = 0.004). Therefore, means of $\dot{V}_{O_2}$ and heart rate were calculated over 15-minute periods of daytime during the unstressed condition where motion levels were similar to those during the stressed condition. To do so, a 15-minute running mean was calculated with the R package CaTool (R Core Team, 2012) throughout the entire daytime period prior to the experiments and all 15-minute periods with similar VeDBA to that recorded during the stressed condition were used to calculate mean $\dot{V}_{O_2}$, heart rate and VeDBA of unstressed with control for similar motion, in incubating birds. Using Shapiro-Wilk test, the populations from which the data were sampled were found normally distributed. As for birds at high activity, Wilcoxon signed-ranks tests and paired t-tests were conducted to test the difference in the overall stress response, using 'unstressed/stressed' data, and in the stress response *per se*, using 'unstressed with control for similar motion/stressed' data. N = 6 in both cases. However only p-value from the t-test are shown to avoid confusion.

Table 3-1 Analysis summary

Aims			Birds type	Statistical analyses	Variables
	Activity level	**Controlling for similar motion**			
Looking at the importance of movements on the cardio-respiratory stress response in king penguins	***High activity***	***No*** => **Overall *stress response***	six birds in courtship (group B)	Paired t-test	***Unstressed:*** Grand mean of $\dot{V}_{O_2}$, heart rate and VeDBA collected during second and third unstressed walking sessions of 10 minutes each. ***Stressed****:* Grand mean of $\dot{V}_{O_2}$, heart rate and VeDBA collected during second and third stressed walking sessions of 10 minutes each.
		Yes => ***Stress response per se***		Paired t-test	As VeDBA was not significantly different between the unstressed and stressed walking session, the stress response *per se* while highly active is the same as overall stress response.
	Low activity	***No*** => **Overall *stress response***	six incubating birds (group C)	Paired t-test	***Unstressed:*** Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the lowest five-minute mean of $\dot{V}_{O_2}$ during the daytime unstressed period. ***Stressed:*** Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the 15-minute stressing session.
		Yes => ***Stress response per se***		Paired t-test	***Unstressed with control for similar motion:*** Grand means of all 15-minutes period of $\dot{V}_{O_2}$, heart rate and VeDBA during the daytime unstressed period, with similar VeDBA as during stressing session. ***Stressed:*** Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the 15-minute stressing session.

3.4 Results

See Appendices for raw data.

Stress responses of high activity birds:

For the high activity birds, the overall stress response was a $\dot{V}_{O_2}$ increase during the stressed condition (P = 0.027) while heart rate and VeDBA did not significantly differ (P = 0.780 and P = 0.23, respectively; Figure 3.2). As VeDBA did not significantly differ for the highly active birds during unstressed/stressed condition (P = 0.23), the overall stress response is the stress response *per se*.

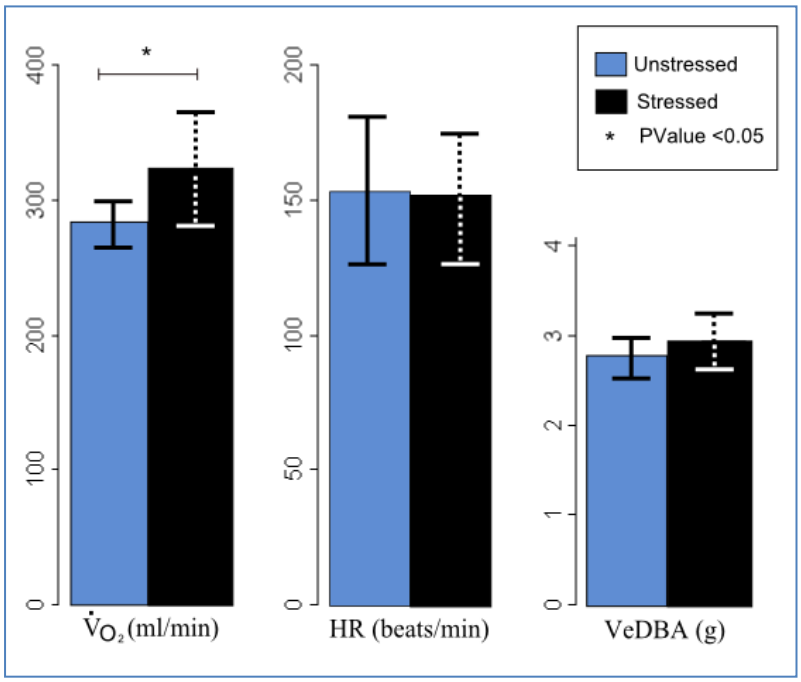


Figure 3.2 Stress responses in high activity birds. Comparisons of 'unstressed (light boxes)/stressed (black boxes)' means of $\dot{V}_{O_2}$, heart rate (HR) and VeDBA, in walking birds. As no change in motion is measured between the unstressed/stressed condition, the overall stress response represents the stress response *per se*. The asterisks represent p-value <0.05 while whiskers represent ± 1 SD.

Stress responses of low activity birds:

Comparisons within the birds at low activity between the stressed and unstressed conditions, without control for similar motion levels, showed a significant increase in $\dot{V}_{O_2}$ and an increase in heart rate during the stressed condition (P < 0.004 and P < 0.002, respectively; light and black boxes of Figure 3.3). However, motion levels also increased during the

stressed condition (P = 0.004). When controlling for motion levels in birds during low activity, $\dot{V}_{O_2}$ remained significantly higher (P = 0.04; dark and black boxes of Figure 3.3) in the stressed condition compared to the unstressed condition, as seen in the 'overall' stress response. However, there was no significant difference in heart rate (P = 0.20) between the stressed and unstressed conditions, showing a variation in the overall and *per se* cardio-respiratory stress response due to motion.

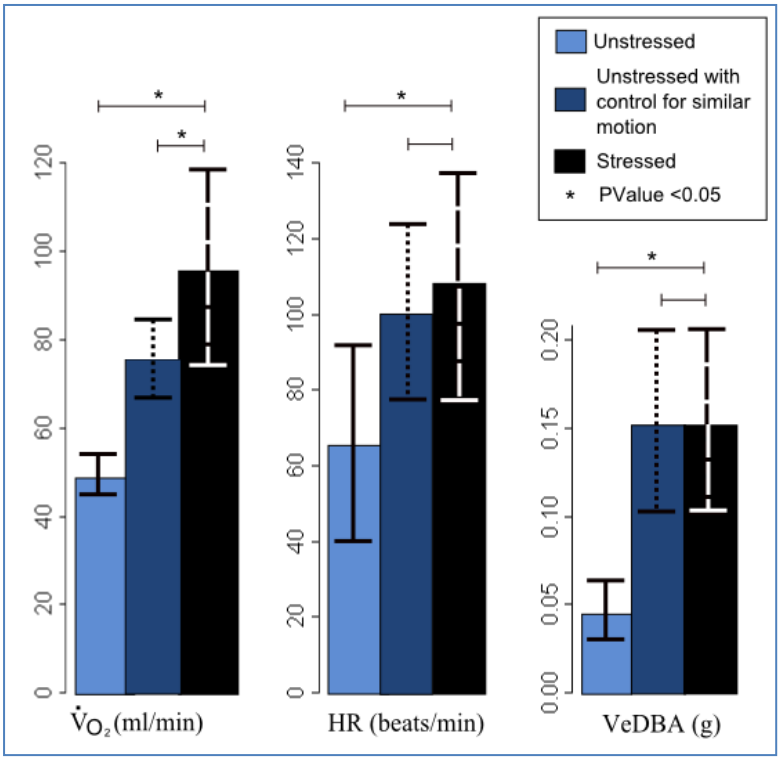


Figure 3.3 Stress responses in low activity birds. Comparisons of 'unstressed (light boxes)/stressed (black boxes)' and 'unstressed with control for similar motion (dark boxes)/stressed (black boxes)' means of $\dot{V}_{O_2}$, heart rate (HR) and VeDBA, in incubating birds. 'Unstressed/stressed' comparison represents the overall stress response while the 'unstressed with control for similar motion /stressed' comparison represents the stress response *per se*. The asterisks represent p-value <0.05 while whiskers represent ± 1 SD.

3.5 Discussion

Stress responses of high activity birds:

As hypothesised, only mean $\dot{V}_{O_2}$ increased in the overall stress response for active birds, while heart rate and VeDBA remained similar (Figure 3.2). As there were no changes in motion between unstressed/stressed conditions, the overall stress response represented the

stress response *per se*, in birds exhibiting a high activity. This increase in metabolic rate represents an increase in physiological function only, such as hormone up-regulation and brain stimulation (Moberg and Mench, 2000, Brener, 1987). Arguably, the initial and forced walking activity, with the precaution to keep a visually fluid walk may have prevented VeDBA from being significantly different during the stressed conditions. However, this should represent the natural stress response of wild king penguins; when disturbed by an anthropogenic stressor, birds in courtship naturally run away (pers. obs.), a behavioural stress response is feasible and matching the experiment protocol. The increase of $\dot{V}_{O_2}$ due to a stressor during high activity has already been shown in fish (Barton and Schreck, 1987). However, no studies measuring the cardiac or the cardio-respiratory response to the presence of a stressor during high activity have been undertaken. The results of the effect of a stressor on heart rate coincided with the hypothesis. As the heart rate has already increased due to the activity level close to maximum (normal range of heart rate in king penguins while onshore is between 50 and 150 beats/minute; Froget et al., 2004) while unstressed, it precluded the heart rate increasing further during the stressed condition. Yet clearly oxygen cannot be consumed by the tissues without delivery and thus, according to Fick's convection equation for the cardiovascular system (Fick, 1870), as heart rate remained constant while $\dot{V}_{O_2}$ increased then either heart stroke volume, the quantity of oxygen per blood volume and/or oxygen extraction by the body tissues has increased. Haemo-concentration shown in response to a stressor (Van Zanten et al., 2004) can be an explanation. Indeed haemo-concentration results in a greater transport of oxygen per unit volume of blood likely serving to increase oxygen extraction.

Stress responses of low activity birds:

The overall stress responses of the birds at low activity levels were in line with the findings of previous studies; when animals were exposed to a stressor during low activity, increases

in mean heart rate and mean $\dot{V}_{O_2}$ were observed (Figure 3.3). Additionally, VeDBA was higher in the presence of a stressor, reflecting the behavioural response of the bird. As mentioned before, the physiological stress response prepares for and enables the behavioural stress response, which is described as 'fight or flight' or the updated 'freeze, flight, fight or fright' and mostly involves movements. However, there is as yet no explanation for the observation that part of this increase in heart rate is 'additional heart rate' (Blix et al., 1974, Stromme and Ingjer, 1978), as calibration of the cardio-respiration system with a gradual intensity of activity was not conducted on these birds. As sex, number of fasting days and reproductive state are known to influence the cardio-respiratory relation, the measurement made with the birds used for the chapter could not be used. When the analysis for birds during low activity was limited to unstressed time periods where the birds were exhibiting similar motion levels as when they were stressed, the observed cardio-respiratory response was an increase only in $\dot{V}_{O_2}$ with no significant increase in mean heart rate, representing an increase in physiological function only to react to the stressor. The results are the first evidence of the short-term cost of the *per se* stress response while freely mobile during low activity behaviours. Additionally, the lack of increase in mean heart rate for similar motion levels contradicts the widely held belief that the cardio-respiratory response to a psychological stressor is principally an increase in mean heart rate. These present findings confirm the supposition that changes in physical motion levels could influence the cardiac stress response (von Borell et al., 2007) and actually demonstrate that the increase of mean heart rate during the stress response is mostly due to the change of motion. Whilst accelerometry may have its limitations (i.e. movement as mastication may not be reflected by VeDBA), its use for measuring overall motion may nonetheless be valid in accurately defining the cardio-respiratory stress response *per se*, leaving the subject free of his behavioural stress response. Indeed, as mentioned before, the description of the normal stress response may also be biased in studies where the protocol minimised the motion of the

subject while stressed, preventing the subject exhibiting its natural behavioural stress response including displacement behaviours which could have helped it to reduce its stress response (Mohiyeddini and Semple, 2013, Wechsler, 1995, Maestripieri et al., 1992).

Together these results show that, in fact, the cardio-respiratory stress response *per se* is different from the overall stress response in freely motile subjects where levels of body motion increase; the former being predominantly an increase in $\dot{V}_{O_2}$ whilst the latter is an increase in both $\dot{V}_{O_2}$ and heart rate. The increase in mean heart rate during exposure to a stressor reported by previous studies appears to be mostly the result of, albeit often small, increases in overall body motion, or at least in striated muscle tone.

Generalisation of the findings

The possibility must be considered that the specific stress response *per se* of king penguins may be different to many species, perhaps due to particular adaptations to their environment. It is known that incubating penguins need to use their energy expenditure efficiently as they are submitted to long fasting periods (Pinshow et al., 1976a, Barrat, 1976, Williams, 1995) and are resting most of their time whilst onshore (Challet et al., 1994, Dewasmes et al., 2001). Thus, incubating king penguins may be in a specific energetic saving mode while resting. This would explain the high $\dot{V}_{O_2}$ increase while stressed, as some physiological parameters, which would have been minimised during resting, may be reactivated during a stress response, even though they are not directly involved in it. Moreover, penguins have a relatively high heart mass in comparison to birds of the same size (Drabek, 1989), which, in accordance with Fick's equation (Fick, 1870) enables them to transport more oxygen per beat relative to other birds due to a greater stroke volume. As demonstrated in humans, a larger heart (more regularly active participants) affects the cardiac stress response by decreasing the increase in heart rate, in comparison to unfit individuals (Vandoornen and

Degeus, 1989). However, the concept of the importance of defining movements (either by defining the initial activity level or the change of motion between unstressed/stressed conditions) in the cardio-respiratory stress response has been shown in the present study. Consequently, future research should specify the stress response as 'overall' or '*per se*' and the levels of activity during which it was measured.

Applications of the findings

Previous studies have never measured the cardio-respiratory response either during high activity or *per se* to a disturbance stressor, while the subject is freely motile. While it would be valuable to validate the generalizability of the present findings to other species, they suggest that in contradiction to the consensus opinion, the stress response *per se* is predominantly an increase in $\dot{V}_{O_2}$, not an increase in mean heart rate. An implication of these findings is that studies that use mean heart rate as an index of stress level are possibly confounded unless motion levels are accurately accounted for. Furthermore, considering the increase in $\dot{V}_{O_2}$ in the stress response *per se*, the question of a potential detrimental effect of an anthropogenic stressor has to be evaluated in terms of effective additional energy expenditure too and not only by heart rate measurements even on a short time scale (Culik and Wilson, 1991, Nimon et al., 1995, Culik and Wilson, 1995). Finally, the mentioned effects on the cardio-pulmonary and behavioural stress response revealed the potential bias of experimental studies, especially in the specific field of studies estimating energy expenditure. Indeed, if the tested animal is not acclimated to the laboratory environment, the referential calibration using the bivariate relationship $\dot{V}_{O_2}$-heart rate or $\dot{V}_{O_2}$-VeDBA may not be correct. To assess how to remove this bias, complementary work has been done on the acclimation of the bird to experimental environment in chapter five, using the results of this chapter as a definition of the cardio-respiratory stress response in king penguins.

4. Avoiding laboratory stress-induced confounds during respirometry: let the king penguin acclimate

After defining the different stress responses in king penguins in chapter three, this chapter measured the time required for subjects to acclimate to the experimental environment and protocol of treadmill walking. This enabled the definition of an adapted protocol to ensure the collection of unbiased data during calibration experiments.

4.1 Abstract

Studies estimating short term energy expenditure of animals are typically laboratory based. To measure the short term energy expenditure of free-ranging animals, proxies, such as heart rate or body movement are usually recorded, using miniature data loggers. These proxies need to be calibrated under laboratory conditions with measurements of $\dot{V}_{O_2}$, while the subject animal is active at different intensities (e.g. on a treadmill). Stress responses are known to affect the cardio-respiratory system; however, no studies of energy expenditure have assessed the acclimation of the animal to experimental conditions and tasks. In addition, as shown in chapter three, a stressor has a clear non-proportional effect on the cardio-respiratory system and behaviour of king penguins (*Aptenodytes patagonicus*), which will add errors to $\dot{V}_{O_2}$-heart rate or $\dot{V}_{O_2}$-VeDBA calibrations. Thus, using a calibration relationship determined from a stressed bird could lead to an overestimation of $\dot{V}_{O_2}$, and subsequently energy expended. The study aimed to (1) define the time needed for the cardio-respiratory system and behaviour of the king penguin to recover post capture and to acclimate to the experimental environment and (2) define the time needed to acclimate to the treadmill walking protocol. To do so, heart rate and VeDBA of six incubating king penguins were recorded while in their colony for more than two hours and then, in conjunction with $\dot{V}_{O_2}$, while undisturbed for more than 10 hours in the laboratory. Furthermore, $\dot{V}_{O_2}$, heart rate and VeDBA of six king penguins in courtship were measured while walking three times on a treadmill for 10 minutes. The results suggest that an incubating king penguin needs on average of a 90 minutes resting time to acclimate to the experimental environment, while birds in courtship walk fluently after one 10-minute session. The results of this study can be used to define an adapted protocol allowing king penguins to acclimate prior to walking-based calibrations, thus minimising the stress-induced error in proxy-based estimates of energy expenditure.

4.2 Introduction

Studies in experimental biology usually involve an artificial and new environment for the subject animals, which often also includes a prescribed activity to be undertaken. This is the case for studies measuring energy expenditure via respirometry. Quantification of energy expenditure is invaluable in answering many questions in biology, particularly in ecological, biomechanical and conservation contexts (Shepard et al., 2008, Halsey et al., 2008d, Arnould et al., 1996, Halsey, 2011, Maloiy et al., 1986). During periods of predominantly aerobic energy metabolism, measurements of the rate of $\dot{V}_{O_2}$ are typically an accurate technique for measuring the estimated energy expenditure (Lighton, 2008). However measuring $\dot{V}_{O_2}$ requires equipment that does not allow the subject animals to range freely in their natural environment. On the other hand, proxy methods can provide accurate estimates of short term energy expenditure in the field, but they require laboratory-based experiments to obtain energy expenditure calibrations relationship (e.g., Green, 2011, Halsey et al., 2011, Portugal and Guillemette, 2011, Froget et al., 2002). A particularly common proxy is heart rate and an increasingly more popular proxy is the measurement of acceleration (Plasqui et al., 2005), from which an index of body movement is commonly derived (ODBA or VeDBA ; 'Overall' or 'Vectorial' 'Dynamic Body Acceleration'; Halsey et al., 2009b, Halsey et al., 2009a, Qasem et al., 2012).

However, such calibration experiments implicitly assume that the behaviours and respiratory-cardiovascular physiology exhibited by the animals in the laboratory are representative of their free-ranging state, but for a number of reasons this may not be the case. Some reviews of these techniques (Gleiss et al., 2010, Green, 2011, Halsey, 2011), and other primary studies (McPhee et al., 2003, Groscolas et al., 2010) discussed issues associated with stressed animals during calibration experiments, which may lead to inaccurate estimations of energy expenditure. Nephew et al. (Nephew et al., 2003)

demonstrated that cardiac (heart rate), behavioural ("fight or flight", Cannon, 1929; response or the updated "freeze, flight, fight or fright" response, Bracha et al., 2004, which can be described by VeDBA) and hormonal (which indirectly modulates $\dot{V}_{O_2}$) stress responses were independent. However, little is actually known about the effects of being stressed on the relationship between $\dot{V}_{O_2}$ and heart rate or $\dot{V}_{O_2}$-and VeDBA. In a recent study of fasting king penguins (*Aptenodytes patagonicus*), Groscolas et al. (Groscolas et al., 2010) reported a higher estimation of energy expenditure for a given measure of heart rate based on the calibrations obtained from 'unstressed' resting birds, compared to when using a calibration derived from birds exposed to a (more typical) graduated treadmill protocol, as reported in Fahlman et al. (Fahlman et al., 2004) (see points B and C in Figure 4.1). To estimate the energy expenditure of unstressed king penguins, Groscolas et al. (Groscolas et al., 2010) used fasting incubating birds in their colony as well as birds in courtship which were maintained in a pen. Birds were weighed every four days from capture until the next shift, and an estimation of their energy expenditure was calculated from their body mass loss, using the linear relationship between endogenous energy stores and body mass of king penguins during fasting phase II (during which the penguins are catabolising predominantly their fat stores; Groscolas et al., 2010). The proposed explanation for this difference in the relationship between $\dot{V}_{O_2}$ and heart rate between the two calibration methods was that the treadmill calibrations induced stress responses in the subject birds, which increased heart rate, resulting in underestimations of energy expenditure for a given measure of heart rate recorded in the wild (Groscolas et al., 2010). This hypothesis was in accordance with the 'additional heart rate response to a stressor' already observed in humans (Blix et al., 1974, Stromme and Ingjer, 1978) and dogs (Boerth et al., 1969, Langer et al., 1979) (Graph in previous chapter Figure 3.1). However, the previous chapter of this thesis reported that the stress response *per se* (i.e. the stress response of the bird as a direct result of the stressor and not including the physiological response to increased body motion as a result of the stressor)

in king penguins corresponded to an increase in $\dot{V}_{O_2}$ only. This 'additional heart rate' observed in the Fahlman et al. (2004) calibration in comparison to the Groscolas et al.

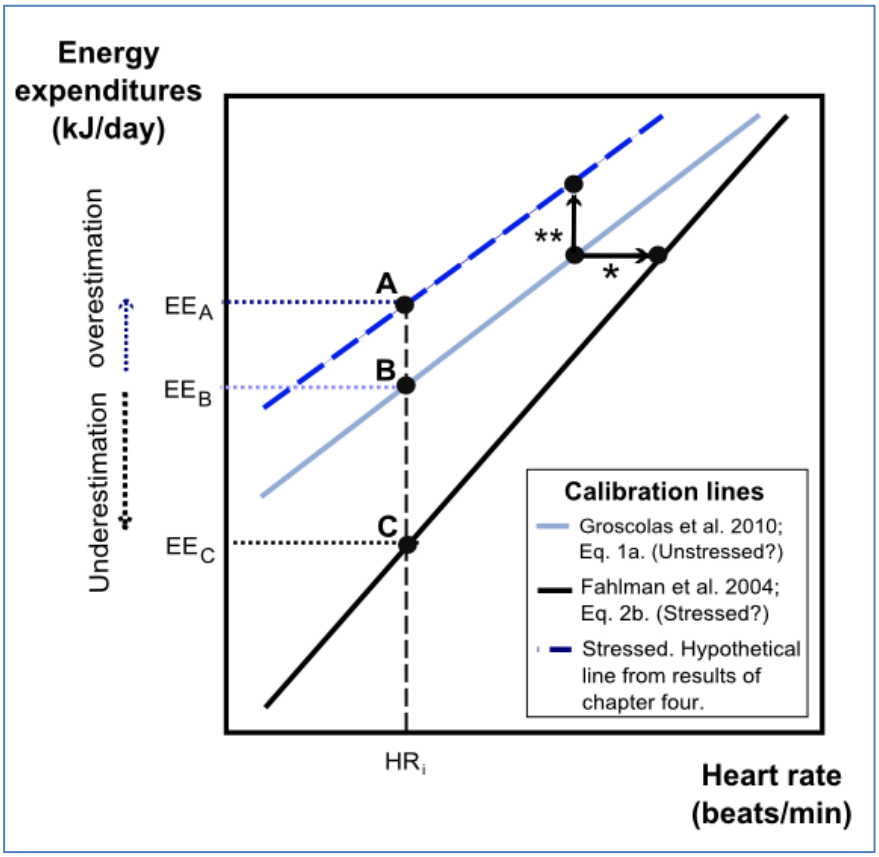


Figure 4.1 Energy expenditure (EE)-heart rate (HR) calibrations for wild king penguins, some of which are potentially stressed-biased. Legends: Calibration from Groscolas et al. 2010; Eq. 1a. (light plain line) is derived from birds monitored while in the colony or kept undisturbed in pens. Calibration from Fahlman et al. 2004; Eq. 2b. (black) has been made with the birds monitored during a typical calibration experiment. The dashed dark line is for a hypothetical stressed-biased calibration from the results of chapter three. Three different EE estimations (dotted lines) are made from Groscolas et al. 2010 (estimation B), Fahlman et al. 2004 (estimation C) and the hypothesis (estimation A) calibrations, using the same HR (dashed line). Groscolas et al. (2010) hypothesised the calibration in Fahlman et al. (2004) to be stressed-biased referring to the 'additional heart rate' (represented as the arrow with the single asterisk) described by Blix et al. (1974) and leading to the underestimation of EE of a wild king penguin from measure of heart rate. Data from chapter three showed that the stress response *per se* is an 'additional metabolic rate', leading to the overestimation of EE (represented as the arrow with the double asterisk).

(2010) calibration relationship may be due to factors other than animal stressed state. Indeed the birds used in these two studies differed in the activities they exhibited (Groscolas et al. 2010 measured resting birds while Fahlman et al. 2004 measured birds walking on a treadmill), as well as the scale of measurement (days, Groscolas et al. 2010; minutes,

Fahlman et al. 2004). However, Groscolas et al (2010) was one of the first studies to try to avoid a stressed-biased calibration relationship. Results from chapter three showed that one of the effects of stress was to elevate $\dot{V}_{O_2}$ for a given heart rate and/or value of VeDBA (for incubating birds or walking birds in courtship), which may consequently lead to an overestimation of true energy expenditure of king penguins in the field (see Point A in Figure 4.1 and Figures 8.3 and 8.4 in the Appendices made with the data collected for this thesis.).

Calibrations of heart rate or body acceleration have already been used on several species including penguins, shags and fish (Blix et al., 1974, Turner and Carroll, 1985, Halsey et al., 2011, Enstipp et al., 2005, Halsey et al., 2007b, Fahlman et al., 2004), see Green, 2011 for a definitive list until 2011). However few such studies considered and tested the required acclimation time needed for the subject animals to become relatively stress-free. Indeed, an animal does not stay constantly stressed, but the stress response attenuates as the animal acclimates. Romero (2004) described the negative feedback of stress mechanisms to avoid noxious effects of long-term stress responses, as well as acclimation to a repeated mild stressor (Romero, 2004). In the context of energy calibration experiments, several stressors may occur: first the bird needs to recover from the stress response due to capture and handling, second it needs to acclimate to the novelty of the experimental environment, and finally it also needs to undertake the exercise protocol e.g. walking on a treadmill or swimming in a flume. The heart rate of king penguins has been observed to recover back to resting levels about an hour after manipulation (Groscolas personal communication). A more recent study from Viblanc et al. (Viblanc et al., 2012a), in which non-acclimated king penguins were restrained for three minutes, showed that post-stressor heart rate was back to pre-stressor levels less than 15 minutes after capture. However, no studies have defined the acclimation time needed for $\dot{V}_{O_2}$ to recover to pre-stressor levels. Protocols of previous

calibration experiments usually enabled the bird to rest in the experimental environment for a minimum of one hour, apparently based on the observation that $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$ (rate of carbon dioxide produced) was steady by this time (Green et al., 2001, Fahlman et al., 2004, Halsey et al., 2007b). Additionally, although behavioural acclimation to walk on a treadmill occurs within just a few training sessions at most (pers. obs.), no studies have measured changes in acceleration patterns during walking sessions. Such information is relevant for assessing the required time for a king penguin to become not only familiar but also acclimated to the task of walking on a treadmill, reflected by a visually natural and fluid walk.

The current experiment aims to quantify the cardio-respiratory and behavioural (i.e. movement) acclimation of king penguins to a calibration experiment by quantifying: (a) the time needed to acclimate to the experimental environment and (b) the minimal number of training sessions required for the birds to be acclimated to the experimental protocol of walking on a treadmill. To do so, heart rate and VeDBA of six incubating king penguins were recorded while in their natural environment for more than two hours and then those measurements and also $\dot{V}_{O_2}$ were recorded while the birds were undisturbed for more than 10 hours in the experimental environment. Additionally, $\dot{V}_{O_2}$, heart rate and VeDBA of six king penguins in courtship were monitored while walking for three sessions of 10 minutes on a treadmill. It is anticipated that the findings from this research will be useful for developing an adapted protocol for acclimating king penguins to calibration experiments, minimising stress-based confounds on measures of cardio-respiratory and locomotion. We hypothesised that the bird would need less than two hours to get acclimated to the experimental environment as well as to get used to the experimental protocol after one walking session.

4.3 Materials and methods

4.3.1 Birds and experimental protocol

4.3.1.1 Acclimation to the experimental environment

Incubating birds (group C; Table 2-1) were hooded to calm them, marked and then equipped *in situ* with the two data loggers (heart rate data logger § 2.1.6, and triaxial acceleration data logger § 2.1.7.). The egg of the bird was simultaneously replaced by a plaster dummy egg and the real egg placed in an incubator at 37.5°C and 60% relative humidity. Subsequently, heart rate and accelerometry were recorded for a minimum of two hours while inside the colony (Figure 4.2 for an example of the experiment schedule). Then, the bird was transferred by hand, still in incubating posture with the dummy egg held against the brood patch, into a respirometer chamber located in a laboratory less than 20 m from the bird's nesting site. It was then left alone overnight for at least 10 hours, while $\dot{V}_{O_2}$, heart rate and VeDBA were monitored. The following day, the bird was submitted to four stressing periods of 15 minutes, with a resting period of one hour in between which was deemed sufficient time to remove any stress effects from previous experiments (Groscolas pers. obs.). $\dot{V}_{O_2}$, heart rate and VeDBA were continuously measured. The unstressed period consisted of leaving the bird alone in the respirometer while incubating its "egg", without any additional noise aside from the noise of the colony. The stressed data are defined as the data collected during a session of 15 minutes with the presence of a stressor (§ 2.2.2.3). The bird was subsequently transferred back to its original location in the colony, with its egg returned. It was observed for the following three days to ensure that it did not desert the egg.

4.3.1.2 Acclimation to the experimental protocol

A bird in courtship (from group B; Table 2-1) was taken from its pen, and was instrumented with the two data loggers (heart rate data logger § 2.1.6, and triaxial acceleration data logger § 2.1.7.). The bird was then placed in the respirometer chamber. The chamber was mounted upon a treadmill such that the birds walked at a controlled speed. $\dot{V}_{O_2}$ (§ 2.1.5.3), heart rate

and VeDBA were measured, while the bird was subjected to two sets of three walking sessions at a speed of 1.4 km/h, each of 10 minutes duration and separated by 10 minutes rest (Figure 2.18 for an example of the experimental schedule). One set was performed with the presence of a stressor and the second set was performed in a quiet environment, i.e. while unstressed. The order of the sets was randomised. Before the unstressed set, the bird was allowed to rest for one hour. The stressed/unstressed conditions were similar to those of the incubating birds, except the birds were walking on a treadmill. After the experiment the birds were released at the same place in the colony from where they had been caught.

4.3.2 Data processing and statistical analysis

4.3.2.1 Acclimation to the experimental environment:

Is acclimation achieved?

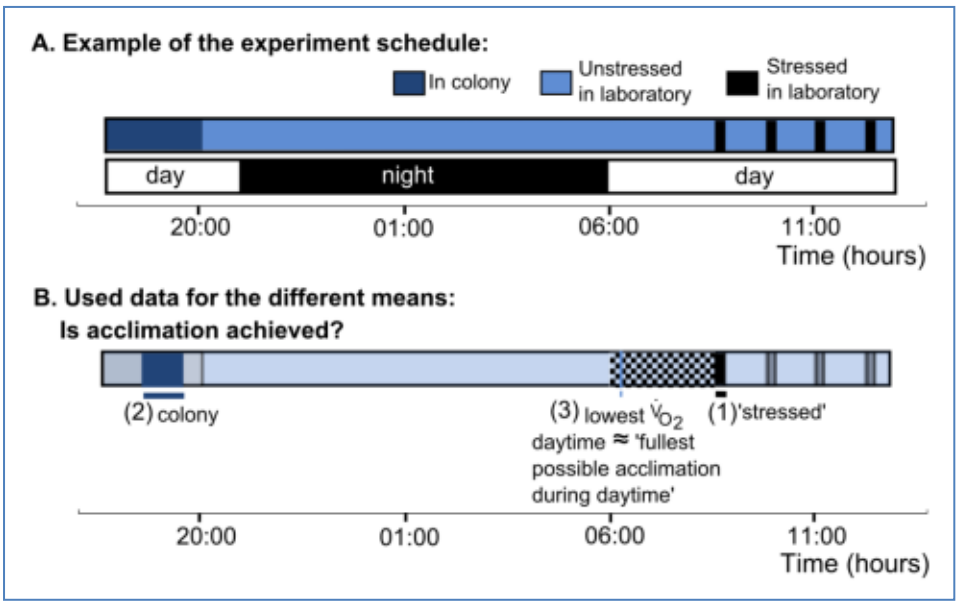


Figure 4.2 A. Example of the schedule of experiment I for an incubating bird (see § 2.2.2.2). The dark area represents when the bird is monitored inside the colony. The light area indicates when the bird is in the respirometer chamber during the unstressed conditions, while the black areas indicate when the bird is submitted to a stressor (see § 2.2.2.2). Day and night times are represented in the second bar. **B. Schedule to assess if acclimation is achieved?** The 'fullest possible acclimation during daytime' are from the lowest $\dot{V}_{O_2}$ obtained during daytime (i.e. 'lowest $\dot{V}_{O_2}$ daytime') selected from data in the black checkered area. The data collected when in the colony are represented by the dark box, and when stressed are represented by the black area. See text for further details.

It was not possible to conduct a hormonal analysis, thus to determine the presence of stressed state (1) $\dot{V}_{O_2}$, heart rate and movement measures from stressed king penguins, measured in chapter three, were used as the indices of stress-biased data, while (2) the heart rate and VeDBA of king penguins in their colony were measured to reflect heart rate and levels of body movement at a level of stressed state typical within the natural context of a breeding colony. (3) The cardio-respiratory stress response *per se* in king penguins is most clearly represented by an increase in $\dot{V}_{O_2}$. Therefore the lowest $\dot{V}_{O_2}$ and the associated heart rate and VeDBA measured during the daytime in the experimental environment were considered to represent the minimum stressed state obtainable (see Figure 4.2). These data were then considered to represent and were termed the 'fullest possible acclimation during daytime', where 'acclimation' refers to physiology and behaviour exhibited with stressor-induced bias removed. Comparison of mean values from (3) with mean values from (2) and (1) shows the level of acclimation of the cardio-respiratory and body movement between natural and experimentally-induced stressed levels.

Means of $\dot{V}_{O_2}$, heart rate and VeDBA of incubating birds while stressed were taken from chapter three. Means of heart rate and VeDBA while birds were incubating in their natural environment were calculated from data of at least two hours duration. As previous observations suggest that king penguins recover from handling within approximately one hour (Groscolas' personal observations of heart rate data, and a standard used protocol from previous studies measuring $\dot{V}_{O_2}$of penguins; Halsey et al., 2007b, Green, 2001, Fahlman et al., 2004), the first hour of data after the handling was not included in analysis, and neither was the period while the bird was manipulated and moved into the respirometer chamber. The displacement process took less than 10 minutes, but a period of 30 minutes subsequent to this was ignored during analysis to ensure any anthropogenic biases were removed (see dark area in Figure 4.2.B). Further to this, in some cases, the bird was being observed before

its removal from the colony, which may have led to a degree of heightened stressed state for the bird if the researcher was spotted. Means of $\dot{V}_{O_2}$, heart rate and VeDBA were calculated for the five minutes of lowest mean $\dot{V}_{O_2}$ during the daytime without the stressor (i.e. checkered area on Figure 4.2.B) while in the respirometer chamber. This enabled standardisation for the effects of circadian rhythms on metabolic rate (Halsey et al., 2008b), since data from the colony or while stressed were measured during daylight hours. Running means of five minute periods derived from a second by second were calculated through the daytime unstressed period using the CaTools package in R Cran (R Core Team, 2012). Then, the lowest five minutes of $\dot{V}_{O_2}$ during the daytime was selected. These means were considered to represent the means at the 'fullest possible acclimation during daytime'. Percentage increases or decreases from these data to the data from the colony and to the data while stressed were calculated. For example, relative to the colony data:

$$Percentage\ VeDBA = \left((VeDBA_{Lowest\dot{V}_{O_2}daytime} - VeDBA_{Colony})/VeDBA_{Colony}\right) * 100$$

; where $Lowest\dot{V}_{O_2}daytime$ is the lowest five minutes of $\dot{V}_{O_2}$ during daytime, and $colony$ represent data from the colony. A Shapiro-Wilk test was used to assess the normality of the populations from which the data were sampled. To determine whether the birds became progressively less stressed in laboratory conditions, $\dot{V}_{O_2}$, heart rate and VeDBA during the period of fullest possible acclimation during daytime were compared to values when the birds were experimentally stressed using a paired t-test. To compare the difference in heart rate and VeDBA while in the colony with those while in the experimental condition at the fullest possible acclimation during daytime, a paired t-test was conducted. The population data were normally distributed; n=6 in all cases.

Calculating time to acclimation:

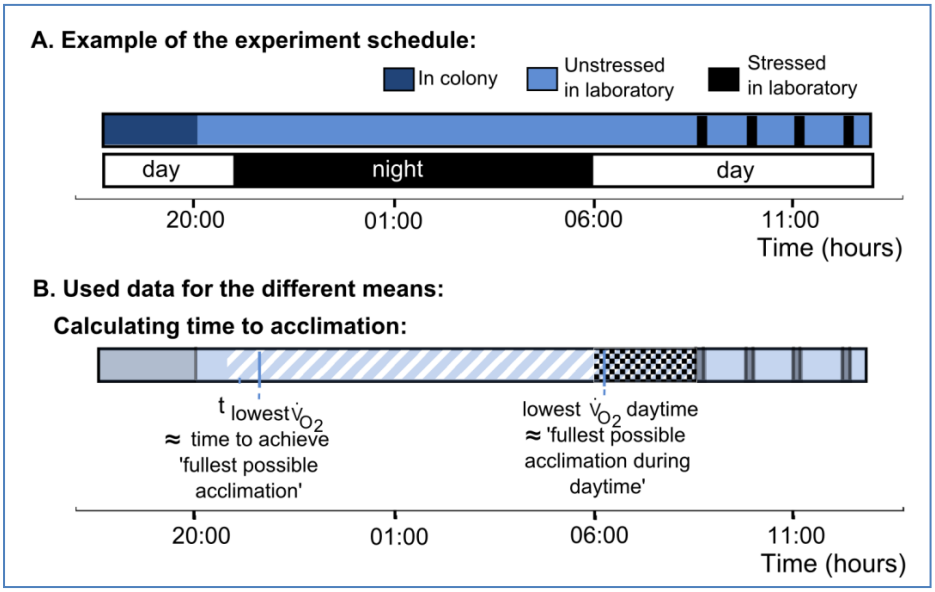


Figure 4.3 A. Example of the schedule of experiment I for an incubating bird (see § 2.2.2.2). The dark area represents when the bird is monitored inside the colony. The light area indicates when the bird is in the respirometer chamber during the unstressed conditions, while the black areas indicate when the bird is submitted to a stressor (§ 2.2.2.2). Day and night times are represented in the second bar. **B. Schedule of the used data for 'Calculating time to acclimation'**. The data at 'fullest possible acclimation during daytime' are from the lowest $\dot{V}_{O_2}$ obtained during daytime (i.e. 'lowest $\dot{V}_{O_2}$ daytime') selected from data in the black checkered area. The time to reach the 'fullest possible acclimation' is the time t with the lowest $\dot{V}_{O_2}$ selected in the white striped area. See text for further details.

The time in the experimental environment taken to reach lowest $\dot{V}_{O_2}$ ('$t_{lowest\dot{V}_{O_2}}$) was calculated for each individual, again based on a five-minute running mean derived second by second throughout the unstressed period. Although physiological parameters are influenced by circadian rhythms (Halsey et al., 2008b), the entire unstressed period of 10-14 hours, including hours at night (white striped area from Figure 4.3.B), was analysed. The aim of this analysis was to find the time needed by the birds to reach minimum metabolic rate which, in the experimental environment, represents the fullest possible acclimation of the bird and thus its acclimation time. Unfortunately as the bird was usually placed in the respirometer at the start of the evening, the time of the fullest possible acclimation might occur during the night, when $\dot{V}_{O_2}$ tends to be lower. Consequently, selecting the minimum metabolic rate during only the daytime could bias the results. Thus, a preliminary analysis was made to select the lowest five-minute mean $\dot{V}_{O_2}$ of the entire experimental period and

checked that it did not occur during the period of naturally minimum metabolic rate for king penguin (between 1h00 and 3h00 from heart rate data; Halsey et al., 2008b). The median, instead of the mean, of the '$t_{lowest\dot{V}_{O_2}}$' for each bird was calculated due to the small sample size and the non normal distribution of the data. The results showed that lowest $\dot{V}_{O_2}$ was typically reached after approximately 90 minutes, see § 4.4.1. Mean data obtained from five-minute lowest $\dot{V}_{O_2}$(i.e. at t= $t_{lowest\dot{V}_{O_2}}$) were taken as the fullest possible acclimation and were compared with fullest possible acclimation during daytime by paired t-tests to test their similarity.

Testing the different acclimation times

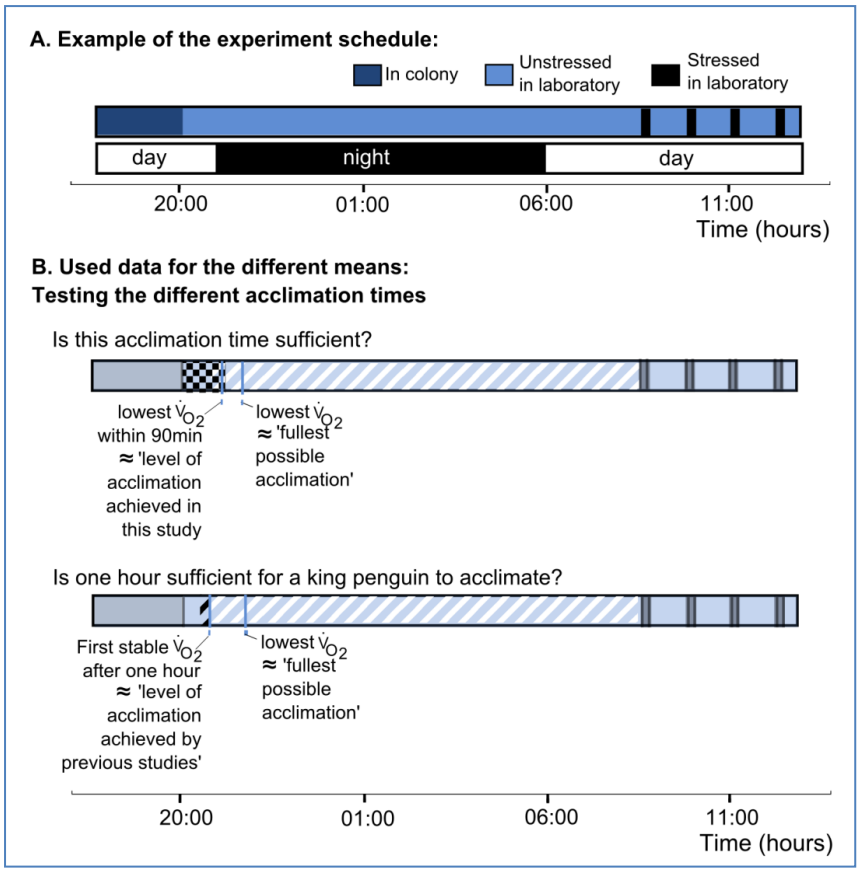


Figure 4.4 A. Example of the schedule of experiment I for an incubating bird (see § 2.2.1.2). The dark area represents when the bird is monitored inside the colony. The light area indicates when the bird is in the respirometer chamber during the unstressed conditions, while the black areas indicate when the bird is submitted to a stressor (see chapter 2.2.1.2). Daytime and night times are represented in the second bar. **B. Schedule of the used data for 'testing the different acclimation times'**. The 'fullest possible acclimation' is from the lowest $\dot{V}_{O_2}$ selected in the white striped area. The data of the 'level of acclimation achieved in by previous studies' (i.e. first stable $\dot{V}_{O_2}$ after one hour) are selected from black striped area. See text for further details.

Is this acclimation time sufficient? To ensure that this acclimation time of 90 minutes (§ 4.4.1) is sufficient to remove any cardio-respiratory and behavioural bias, the lowest mean $\dot{V}_{O_2}$ over five minutes during those 90 minutes (i.e. acclimation time from this study; black checkered area, Figure 4.4) was calculated for each bird. For some birds, this value was equal to that representing the fullest possible acclimation across the entirety of the experiment. These means were considered to represent level of acclimation achieved using the protocol found from this study. A paired t-test compared these $\dot{V}_{O_2}$, heart rate and VeDBA values to those representing the fullest possible acclimation to test whether minimum metabolic rate obtainable in the laboratory within 90 minutes is similar to that obtainable over many hours.

Is one hour sufficient for a king penguin to acclimate? The previous protocol used to acclimate penguins included one resting hour prior to the experiment (Groscolas' pers. obs. for heart rate and (Halsey et al., 2007b, Green, 2001, Fahlman et al., 2004) for $\dot{V}_{O_2}$). In these studies, the penguin was considered acclimated if heart rate or $\dot{V}_{O_2}$ had been stable for the previous few minutes or 20 minutes, respectively. To assess this protocol, $\dot{V}_{O_2}$, heart rate and VeDBA values obtained after one hour in the experimental environment were analysed. A mean of five-minute of the values, subsequent to the first 20-minutes of stable measures of $\dot{V}_{O_2}$, was calculated (see Figure 4.4), and taken to represent the level of acclimation achieved by previous studies. To determine if the previously used protocol was sufficient to allow the bird to acclimate, a paired t-test was conducted on the mean data representing the levels of acclimation achieved by previous studies and the data at the fullest possible acclimation. The population data were normally distributed as demonstrated by a Shapiro-Wilk test; n=6 in all cases.

Are the data using the acclimation protocol of previous studies and from this study similar? Finally, mean data at the level of acclimation achieved by previous studies (first

five-minute with previous 20-minutes of stable measures of $\dot{V}_{O_2}$ after one hour) and the mean data of level of acclimation achieved using the protocol found from this study (data with the lowest $\dot{V}_{O_2}$ achieved in 90 minutes) were compared with a paired t-test (Figure 4.5).

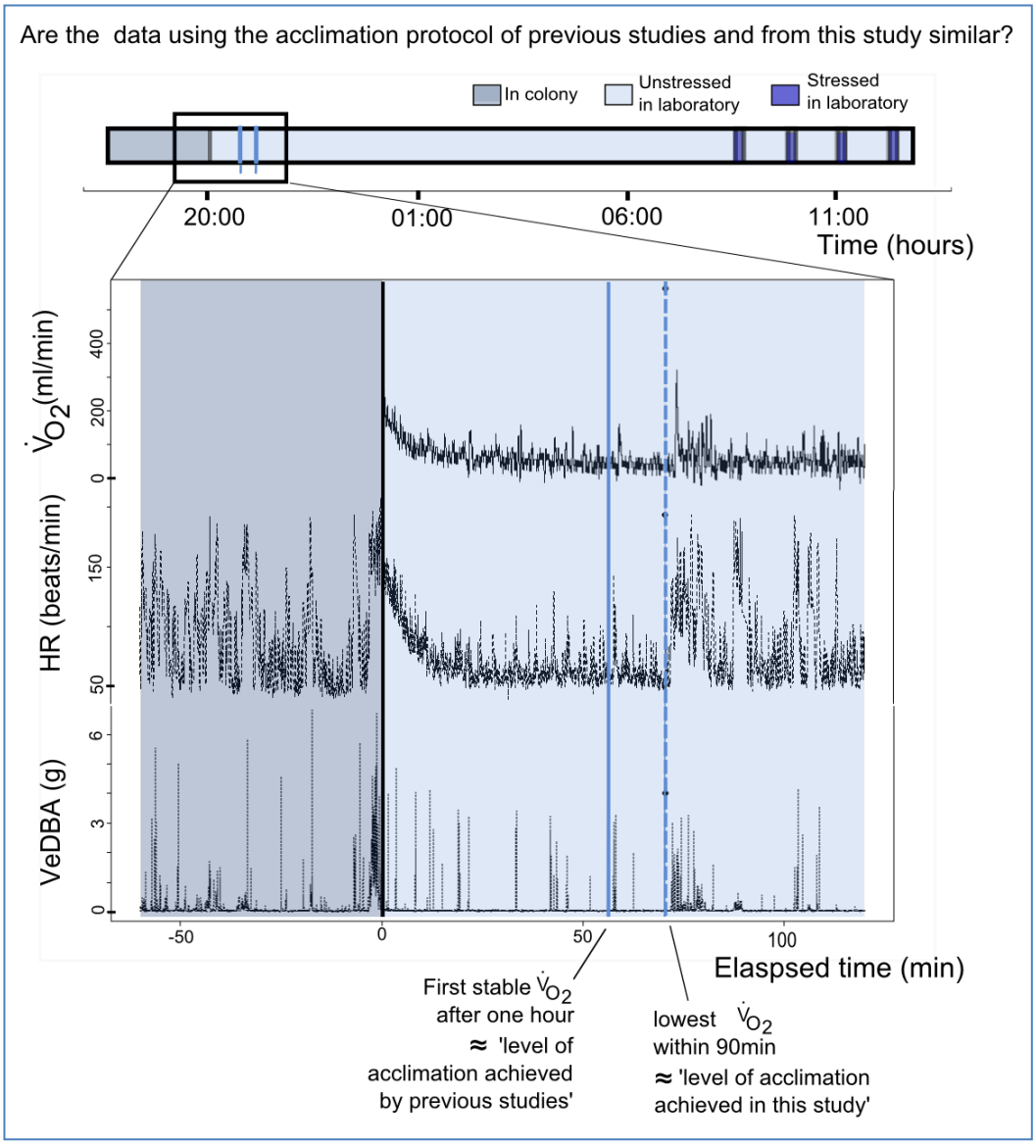


Figure 4.5 Detailed representations of $\dot{V}_{O_2}$, heart rate (HR) and VeDBA data used for the aim of 'Are the data using the acclimation protocol of previous studies and from this study similar?' Sample $\dot{V}_{O_2}$, heart rate (HR) and VeDBA data for an incubating king penguin in the colony (dark shading) and in a respirometer chamber (light shading). $\dot{V}_{O_2}$, heart rate (HR) and VeDBA are represented as a function of time (min). t=0 (plain black vertical line) indicates when the bird was placed in the respirometer chamber. When t<0, the penguin is present in the colony. The light plain line is the time t of the first stable $\dot{V}_{O_2}$ one hour subsequent to being placed in the respirometer chamber. The dashed line is the time t of the lowest $\dot{V}_{O_2}$ for this specific bird (t ≃ 70 min).

4.3.2.2 Acclimation to the experimental protocol:

Acclimation during walking sessions: The means of $\dot{V}_{O_2}$, heart rate and VeDBA for each walking session undertaken by the birds in chapter three (see § 2.1.2.1 and § 2.2.2.1) were also used in the present chapter. ANCOVAs were used to test the number of walking sessions needed before the acclimation of these three variables on each bird individually during the first to third walking sessions. The package 'lme4' in R Cran was used (R Core Team, 2012) to run linear models of the following form: e.g. $\dot{V}_{O_2}$ = walking session + individual [random]. If walking session was significant, post hoc paired t-tests with p-value adjusted as Holm, Bonferroni and without adjustment, were conducted. The population data were normally distributed as demonstrated by a Shapiro-Wilk test; n=6 in all cases.

Acclimation within the first walking session*:* Means of two consecutive four-minute time intervals during the 10-minute walks were calculated. Four-minute durations are sufficient to enable comparison of stabilised contemporary means of each measured physiological parameter. The first minute of the session was removed as the response time of $\dot{V}_{O_2}$ has been shown to occur within one minute (See General Methods chapter § 2.3.1.1). These time intervals were thus defined as from minute two to five inclusive and from minute six to nine inclusive. To compare the acclimation of each parameter within the first walking session, paired t-tests for the means of $\dot{V}_{O_2}$, heart rate and VeDBA were conducted between the two four-minute time intervals of the first walking session. The population data were normally distributed using Shapiro-Wilk test; n=6 in all cases. Results were considered to indicate a significant effect where $p<0.05$.

Table 4-1 Analyses summary

Aims				Birds type	Statistical analysis	Variables
Removing stress-induced confound from cardio-respiratory and movement data	**Acclimation to experimental environment (laboratory)**	***Is acclimation achieved?***		Six incubatings male (group C)	Paired t-test	• *Stressed:* Means of $\dot{V}_{O_2}\dot{V}_{O_2}$, heart rate and VeDBA while stressed for 15 minutes per individual (data from chapter three). • *Colony:* Means of heart rate and VeDBA while inside the colony per individual (30 minutes of data minimum). • *Fullest possible acclimation during daytime:* Means of HR and VeDBA of the lowest five-minute mean of $\dot{V}_{O_2}$ in day time only (i.e. $lowest\dot{V}_{O_2}daytime$).
		Calculating time to acclimation			Median ± median absolute deviation	*'Acclimation time t':* Elapsed time to reach the lowest five-minute mean of $\dot{V}_{O_2}\dot{V}_{O_2}$ in all resting period after being place in the per individual (i.e time of $lowest\dot{V}_{O_2}$).
			Are the fullest possible acclimation during daytime and fullest possible acclimation similar?		Paired t-test	• *Fullest possible acclimation during daytime:* Means of HR and VeDBA of the lowest five-minute mean of $\dot{V}_{O_2}$ in day time only (i.e. $lowest\dot{V}_{O_2}daytime$). • *Fullest possible acclimation:* Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the lowest five-minute mean of $\dot{V}_{O_2}$ in all resting period per individual (i.e. $lowest\dot{V}_{O_2}$).
		Testing the different acclimation times.	*Is this acclimation time sufficient? (90 minutes)*		Paired t-test	• *Fullest possible acclimation:* Means of $\dot{V}_{O_2}\dot{V}_{O_2}$, heart rate and VeDBA of the lowest five-minute mean of $\dot{V}_{O_2}$ in all resting period per individual (i.e. $lowest\dot{V}_{O_2}$). • *Data at the level of acclimation achieved by using the protocol found in this study:* Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the lowest five-minute mean of $\dot{V}_{O_2}$ found within the acclimation time of this study (i.e. $lowest\dot{V}_{O_2}found\ within\ 90min$).
			Is one hour sufficient for a king penguin to acclimate?(previous studies)		Paired t-test	• *Fullest possible acclimation:* Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the lowest five-minute mean of $\dot{V}_{O_2}$ in all resting periods per individual (i.e. $lowest\dot{V}_{O_2}$). • *Data at the level of acclimation achieved by previous studies:* Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the first stable five-minute mean of $\dot{V}_{O_2}$ after one hour per individual.
			Are the data using the acclimation protocol of previous studies and from this study similar?		Paired t-test	• *Data at the level of acclimation achieved by using the protocol found in this study:* Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the lowest five-minute mean of $\dot{V}_{O_2}$ found within the acclimation time of this study (i.e. $lowest\dot{V}_{O_2}found\ within\ 90min$). • *Data at the level of acclimation achieved by previous studies:* Means of $\dot{V}_{O_2}$, heart rate and VeDBA of the first stable five-minute mean of $\dot{V}_{O_2}$ after one hour per individual.
	Acclimation to experimental protocol (walking).	***Acclimation across walking sessions***		Six males in courtship (group B)	Repeated ANCOVA of the mixed model. Post hoc: paired t-test with p-adjusted for Holm , Bonferroni and no P adjusted.	$\dot{V}_{O_2}\dot{V}_{O_2}$ Parameter = order + Individual [random]. Means of $\dot{V}_{O_2}$, heart rate and VeDBA while 15-minute walking session for each session (three means for each parameters) per individual.
		Acclimation during the first walking session			Paired t-test	Means of $\dot{V}_{O_2}\dot{V}_{O_2}$, heart rate and VeDBA during 4-minutes interval from the first walking session (two means for each parameters i.e. from minutes two to five and from minutes six to nine) per individual.

4.4 Results

See Appendices for raw data.

4.4.1 Acclimation to the experimental environment:

Is acclimation achieved? The $\dot{V}_{O_2}$, heart rate and VeDBA in king penguins are all significantly lower during periods of rest compared to periods when a stressor is present in the laboratory (45% , 40% and 67% lower, p= 0.004, 0.002 and 0.004 for $\dot{V}_{O_2}$, heart rate and VeDBA, respectively; see Figure 4.6) which is consistent with the results from chapter three. Additionally, heart rate and VeDBA at the lowest mean $\dot{V}_{O_2}$ during daytime are significantly lower than when the birds are in their natural environment (27% and 61% lower; p=0.03 and p=0.01 for heart rate and VeDBA, respectively; Figure 4.6). For this reason data at lowest mean $\dot{V}_{O_2}$ during daytime were considered to represent the fullest possible acclimation during daytime.

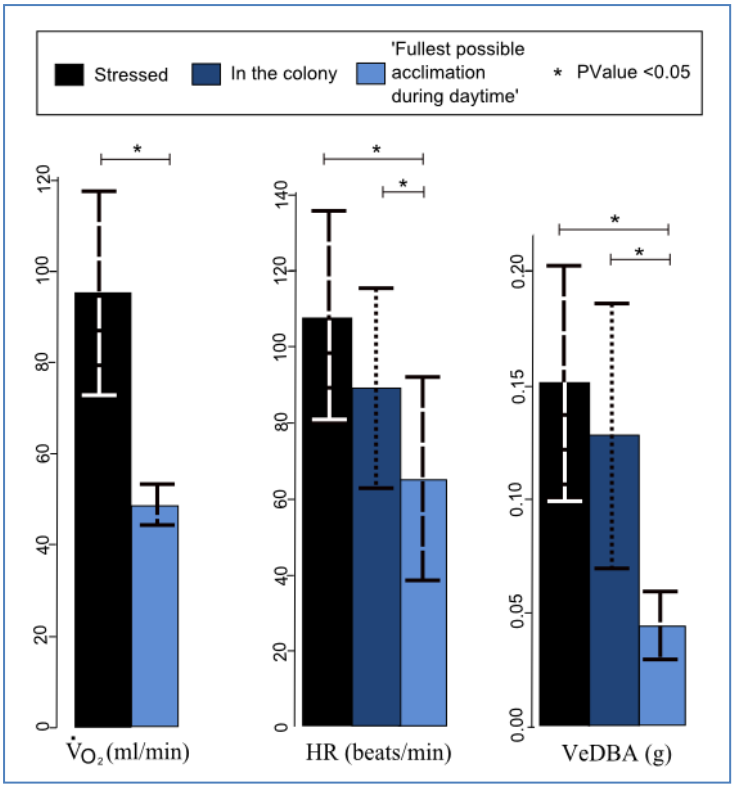


Figure 4.6 Comparison of mean $\dot{V}_{O_2}$, heart rate (HR) and VeDBA of incubating king penguins during periods when the stressor was present (black, from chapter three), periods in the colony (dark box) and once at the fullest possible acclimation during daytime to the experimental environment (light box). The asterisk indicates where p<0.05 while whiskers represent means ± 1 SD.

Calculating time to acclimation: The median time needed to reach the fullest possible acclimation during daytime, subsequent to having been placed in the respirometer, was approximately 1.5 h (92 min ± 30). No significant changes have been shown between data of the fullest possible acclimation and the data of the fullest possible acclimation during daytime (i.e. lowest $\dot{V}_{O_2}$ and lowest $\dot{V}_{O_2}$ during daytime, respectively); however heart rate had a tendency to be lower for the data of the fullest possible acclimation during daytime (p=0.12, 0.06, and 0.25, for $\dot{V}_{O_2}$, heart rate and VeDBA, respectively; Figure 4.7). Due to the small sample size, this difference is worth noting. VeBDA is lower during the day; however, given the magnitude of the scale, this difference represents a minimal physical change.

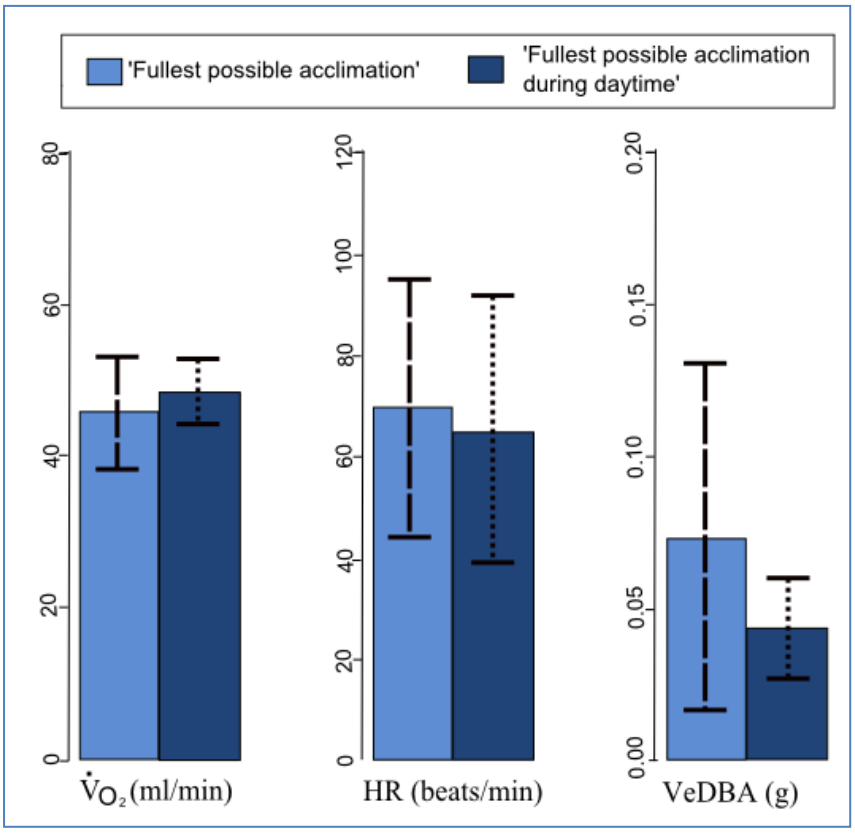


Figure 4.7 Comparison of mean $\dot{V}_{O_2}$, heart rate (HR) and VeDBA of captive incubating king penguins at the 'fullest possible acclimation' (light box) and at the 'fullest possible acclimation during daytime' (dark box). There was no significant difference between the data of the fullest possible acclimation and the data of the fullest possible acclimation during daytime. The whiskers represent means ± 1 SD.

Testing the different acclimation times

Is this acclimation time sufficient to acclimate? Comparison of the data at the level of acclimation achieved in this study (i.e. lowest $\dot{V}_{O_2}$within 90 min) and the data of fullest possible acclimation indicated no significant difference (p= 0.25, 0.47 and 0.34 for $\dot{V}_{O_2}$, heart rate and VeDBA, respectively; Figure 4.8).

Is one hour sufficient for the bird to acclimate? After exposure to the respirometer chamber for one hour, the first stabilised $\dot{V}_{O_2}$ (i.e. protocol used in previous studies to obtain acclimated data) was still significantly higher than the $\dot{V}_{O_2}$ of fullest possible acclimation (P= 0.02), while heart rate and VeDBA were not statistically different (P = 0.98, P= 0.13 respectively; Figure 4.8). These results indicated that the physiogical state of the bird in the laboratory is still heightened after one hour even though measurements have settled.

Are the data using the acclimation protocol of previous studies and from this study similar? Comparison of data while acclimated from both different acclimation protocols (used in previous studies and from this study) showed a significantly lower $\dot{V}_{O_2}$ for the data using the protocol from this study, but no difference regarding heart rate and VeDBA (p= 0.005, 0.19 and 0.15, respectively; Figure 4.8).

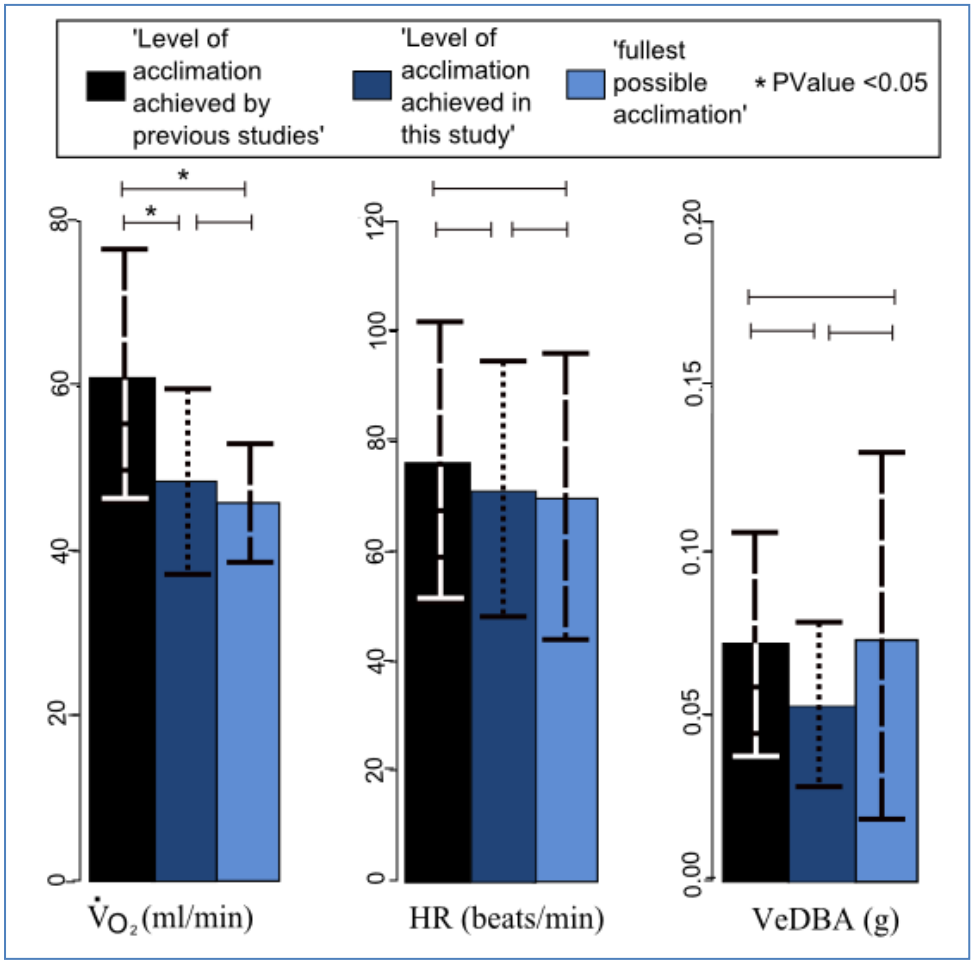


Figure 4.8 Comparison of mean $\dot{V}_{O_2}$, heart rate (HR) and VeDBA of captive incubating king penguins at the 'level of acclimation achieved by previous studies' (black boxes), level of acclimation achieved in this study' (dark boxes) and 'fullest possible acclimation' (light boxes) when inside a respirometer chamber. The asterisk indicates a p-value <0.05 while the whiskers represent means ± 1 SD.

4.4.2 Acclimation to the experimental protocol:

***Acclimation during walking sessions**:* The results of repeated measures ANCOVA indicated a significant decrease in $\dot{V}_{O_2}$ over walking sessions (P=0.02). VeDBA did not significantly differ; however the data do suggest a tendency for VeDBA to decrease across walking sessions (P=0.08). There was no significant change in heart rate between walking sessions (P=0.42). Post hoc paired t-tests for $\dot{V}_{O_2}$ per walking session found no significant differences with any p-value adjustment (P=0.21 and 0.21 for Holm and Bonferoni adjustment,

respectively). There was a tendency for $\dot{V}_{O_2}$ to be greater during the first walking session than during the subsequent sessions but due perhaps to the small sample size this was not significant (using no p-value adjustment, P = 0.07, see Figure 4.9). No changes in VeDBA were found between the different orders of walking sessions (P= 0.55, 0.55, 0.18 for p-value adjustment of Holm, Bonferroni and none, respectively). However, the first session showed a considerably bigger standard deviation for VeDBA.

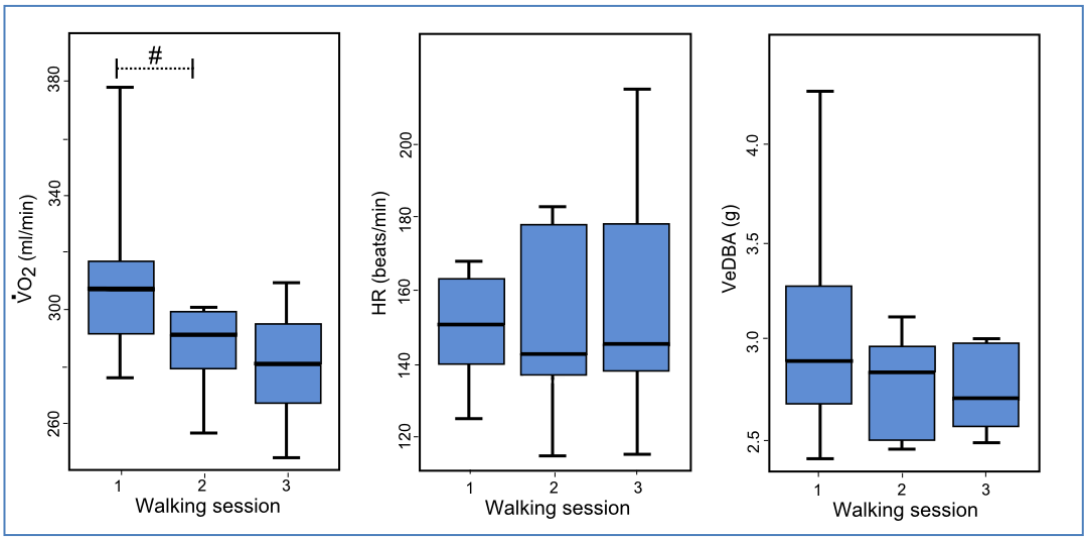


Figure 4.9 Boxplot of $\dot{V}_{O_2}$ heart rate (HR) and VeDBA during the three walking sessions. The black lines represent the median, the light box the 1st and 3rd interquartile range and the whiskers represent the extreme values. The hash sign indicates p = 0.07. The overall results of ANCOVA showed a decrease of $\dot{V}_{O_2}$ across the walking sessions.

Acclimation within the first walking session: Paired t-test comparisons between the two time intervals within the first walking session showed no significant difference (P = 0.17, 0.78, 0.39; for $\dot{V}_{O_2}$, heart rate and VeDBA, respectively).

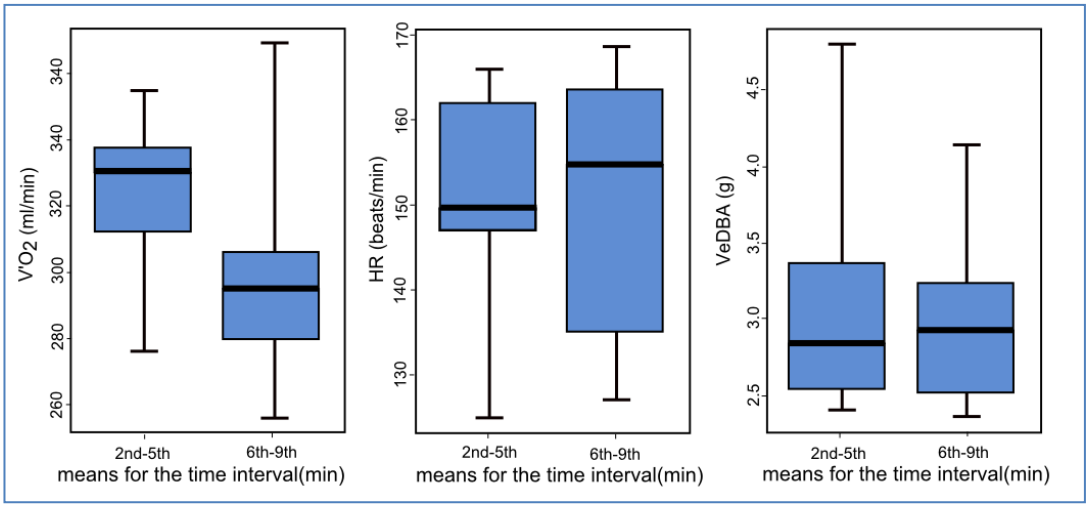


Figure 4.10 Boxplot of mean $\dot{V}_{O_2}$, heart rate (HR) and VeDBA for the two consecutive four-minute time intervals of the first walking session. The black lines represent the median, the light box the 1st and 3rd interquartile range and the whiskers represent the extreme values. There were no significant differences between the different time intervals.

4.5 Discussion

Acclimation to the experimental environment:

The cardio-respiratory and behavioural stress response *per se* of a king penguin is represented by an increase in $\dot{V}_{O_2}$ but not of HR or VeDBA (chapter three). Therefore the lowest $\dot{V}_{O_2}$ and its related heart rate and VeDBA measured in the experimental environment were considered to represent the fullest possible acclimation that is reasonably obtainable in the experimental environment. From this, the acclimation time of incubating king penguins, subsequent to exposure to the experimental environment, is calculated to be about 90 minutes. Although the natural $\dot{V}_{O_2}$ could not be measured and ascertained, the data measured in this study using the acclimation protocol of 90 min can be expected to represent data without stress-contamination, with a good degree of certainty. Indeed, firstly, $\dot{V}_{O_2}$ of the birds decreased constantly over the first few hours that they were in the respirometer, which suggests that the bird does acclimate in the experimental environment. Secondly, as the lowest $\dot{V}_{O_2}$ during daytime is significantly lower than $\dot{V}_{O_2}$ measured during a stressing

experiment (data from chapter three), this showed that birds did not stay highly stressed during the 12-hour resting period. Thus, data from the lowest $\dot{V}_{O_2}$ during daytime can be considered at least as the data of the less stressed state which can be attained in the experimental context, or as data at the fullest possible acclimation during daytime. Thirdly, it has been shown that the $\dot{V}_{O_2}$, heart rate and VeDBA of king penguins all increase during stress in an artificial environment due to the presence of humans (i.e. overall stress response, chapter three). Indeed a stressing situation mostly forces an incubating bird to move, by increasing its displacement behaviour, responding aggressively to the antagonist or trying to flee or at least move further away from the stressor, following the 'fight or flight' behavioural stress response (Cannon, 1929). However, VeDBA during data at the fullest possible acclimation during daytime was 61% lower than VeDBA measured for birds in the colony (for a 27% lower heart rate). This suggests that, at this time, incubating birds were acclimated or less stressed than is typically the case in their natural environment.

Choosing the data with the lowest $\dot{V}_{O_2}$ during daytime or the data with the lowest $\dot{V}_{O_2}$ as references to represent non stressed biased data may be an extreme choice, and in psychological terms at least, the birds could be unstressed before reaching this physiological state. Indeed the data with the lowest $\dot{V}_{O_2}$ are usually used to represent the resting metabolic rate (Halsey et al., 2008b). However, birds often exhibit more active behaviours in the respirometer chamber than simply resting (e.g. being curious and 'exploring' the new environment), and thus, having a higher $\dot{V}_{O_2}$without being stressed. Consequently, the extreme data were chosen for analysis, which ensured having a stress-biased free state of the bird. Thus the present data can be considered as representing an initial assessment of the stress free cardio-respiratory state of a king penguin. Furthermore, these results suggest that a protocol incorporating a period of acclimation of 90 min is needed to ensure that cardio-

respiratory data, for example for heart rate- or accelerometry-$\dot{V}_{O_2}$ calibrations, are affected as little as reasonably possible by stress-related confounds. This protocol contrasts with the previous suggestions and protocols (Groscolas' personal observation for heart rate, and Halsey et al. (2007b), Green (2001, Fahlman et al. (2004) for $\dot{V}_{O_2}$), where king penguins were considered as acclimated in one hour, if $\dot{V}_{O_2}$ had been stable for the previous 20 minutes. The present results show that data from these two different protocols differ regarding obtaining an unbiased $\dot{V}_{O_2}$, suggesting that an error may still remain if an appropriate protocol is not applied.

***Acclimation to the experimental protocol of walking sessions**:*

As shown in chapter three, the birds had a higher $\dot{V}_{O_2}$ while walking in the presence of a stressor; however, there was no change in VeDBA in either experimental condition. The present analyses showed some evidence that the variation in the VeDBA reduced over the course of different unstressed walking sessions. This suggests that the king penguins became acclimated to walking on a treadmill during the first walking session of the day (note that these birds had been pre-selected as relatively good walkers). The high standard deviation of VeDBA of the first walking session in comparison to later walks suggests that some individuals need more time to acclimate to the walk. There was also some evidence that $\dot{V}_{O_2}$ decreased over the three different walking sessions (Figure 4.9). This suggests that a walking session prior to the data collection session is advisable to ensure that the king penguin acclimated to walking on a treadmill, but is not essential. However, it is important to mention that the present results used a previously widely-employed protocol (personal observation of R. Groscolas; Halsey et al., 2007b, Green, 2001, Fahlman et al., 2004), which allowed the bird to acclimate for only 60 minutes. This may have influenced the $\dot{V}_{O_2}$ results of this present study, regarding data of the birds while walking, in the sense that the

significant effect of walking session on $\dot{V}_{O_2}$ may also be due to the unachieved acclimation to the experimental environment.

Potential limitations of this study

King penguins used for energy expenditure measurement are typically birds in courtship, as the birds used for the calibration experiment are forced to be active, which ensures the scientific impact on the studied population is minimised. In this study, the birds used to estimate the acclimation time in the laboratory were incubating birds, which, as mentioned previously, have a different metabolism to bird in courtship (Froget et al., 2001, Green et al., 2001). However, birds in courtship tend to move more while in the respirometer chamber and $\dot{V}_{O_2}$ would therefore have been more related to their activity levels than to their stressed levels. Thus as a first study on acclimation of king penguins to calibration experiments, incubating birds were used despite potential dissimilarity regarding the acclimation of birds in courtship.

Thus, from the results of this first study on acclimation of king penguin, it is suggested an acclimation period of 90 minutes should be used rather than the standard one hour used previously. This should enable an improvement in the accuracy of the measurement of the response of the cardio-respiratory system of king penguins by reducing the influence of stressed state on the data.

4.6 Calibration guidelines

Based on the study findings, an adapted protocol to measure the cardio-respiratory physiology and behaviour of king penguins while walking on a treadmill involves:

a) a resting and acclimating period inside the respirometry chamber of at least 1.5 hours

b) a practice walking session before data collection for calibration relationship on trained king penguins.

These guidelines reduce potential stress-induced confounds on the behavioural and cardio-respiratory systems of king penguins used during calibration.

5. An approach to uncover the cost of pedestrian locomotion: a biomechanical look at the ‘optimised fat penguin’

The parameters influencing the cost of the transport in animals are discussed in this chapter using the cost of transport of king penguin as an example.

5.1 Abstract

The parameters influencing the gross cost of transport (GCOT) are still uncertain. Indeed the same parameters have contradictory effects depending of the species, or when looked inter- or intra-specifically. This is the case for the parameter of body mass. Between species, mass-specific GCOT decreases with an increasing body mass, while GCOT increases with the increase in body mass when compared within one species. An increasing number of studies showed the benefit of partitioning GCOT, and an example is to separate the net cost of transport (NCOT, energy used to move a unit distance) and the postural cost of transport (PCOT, energy to maintain the posture for the locomotion). King penguins have been shown to have an independent NCOT regardless of a body mass increase (i.e. heavy penguins use in absolute terms the same amount of energy to move a unit distance as thin penguins). This shows an optimised adaptation of the cost of load carrying, which could help to uncover the parameters influencing GCOT, via understanding the effect of the different parameters on GCOT's partition: NCOT and PCOT. To understand the mechanism of this optimisation, energy expenditure (i.e. $\dot{V}_{O_2}$) and biomechanical measures (i.e. tri-axial acceleration and 3D video) of ten king penguins, each walking at four different speeds and at four different body masses, were analysed. Furthermore, location of the centre of mass at the two body masses was calculated for two king penguin cadavers. Energy expenditure was found to be independent of body mass, while only step width and its repeatability from all the measured stride parameters (i.e. from length, width, frequency, duration, stance duration, swing duration and duty factor) showed a significant change with body mass. Analysis of the acceleration data for each axis individually indicated no significant differences but the gross body acceleration indicated a difference. Pitch, which indicates forward tilt, was significantly increased and waddling was significantly decreased with loss of body mass. Unfortunately, attempts to measure the location of the centre of mass were not successful and the proposed hypothesis of locomotion of the centre of mass explaining the

'optimisation of fat penguins' could not be tested. Despite the lack of significant difference in gait, this research is a step forward in trying to better understand GCOT, by its partition into NCOT and PCOT, and the specific parameter that enable the optimisation of NCOT while body mass change.

5.2 Introduction

The gross cost of transport (GCOT) is described as the relationship between metabolic rate and speed of walking (Taylor et al., 1970, Halsey et al., 2007b). This relationship has been found to be a linear regression in a majority of species (Taylor et al., 1970, Halsey et al., 2007b) (Equation 5-1).

Equation 5-1

$$EE = a * s + b,$$

; where s is the speed (m/s) and EE the energy expenditure (J/min), usually measured as $\dot{V}_{O_2}$ (in ml/min). Net cost of transport (NCOT) is defined as the energy expenditure per unit distance moved (J/m) and is the slope a of the linear relation. The intercept b minus the resting metabolic rate is defined as the postural cost of transport (PCOT) (Figure 5.1).

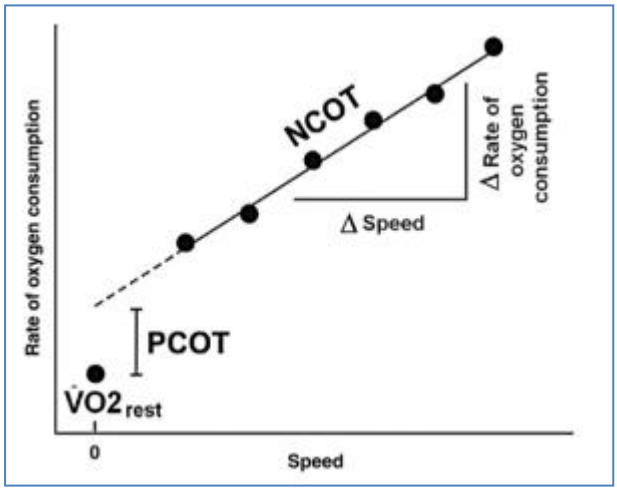


Figure 5.1"Relationship between rate of oxygen consumption ($\dot{V}_{O_2}$) and speed in king penguins walking on a treadmill. Net cost of transport (NCOT) is calculated as the slope of the relationship relating $\dot{V}_{O_2}$to speed for speeds greater than zero. Postural cost of transport (PCOT) is calculated by subtracting resting $\dot{V}_{O_2}$ ($\dot{V}_{O_2}$rest) from the extrapolated y-intercept of relationship describing NCOT." From Halsey et al. (2007b).

The specific parameters which make up the gross costs of pedestrian locomotion are still unclear. The first research on this subject described some important parameters. The cost mainly depends of the pedestrian gait type (walking, running, trotting, gallop, etc. (Heglund and Taylor, 1988, Taylor, 1985, Schmidt-Nielsen, 1972). Besides, an increase in speed requires an increase in energy expenditure as limbs need to move faster, engendered from quicker muscle contractions, reducing the contact time of the feet with the ground per stride cycle, therefore reducing the duty factor (Kram and Taylor, 1990, Fedak et al., 1982, Biewener, 1983). Furthermore, mass-specific comparison of the cost of locomotion across a broad range of species has shown that larger species have a lower mass specific GCOT, as they tend to have a lower stride frequency (Taylor et al., 1970, Kram and Taylor, 1990, Roberts et al., 1998). These observations lead to the conclusion that GCOT is generally linked with the forces needed to support the body's mass and the duration of generating the force necessary to do this, suggesting that the stance phase was the most important parameter influencing the GCOT (Kram and Taylor, 1990, Taylor et al., 1980).

However, recent studies showed that the swing phase was associated with 10 to 26% of the stride cost, revealing its importance in determining GCOT (Marsh et al., 2004, Modica and Kram, 2005, Doke et al., 2005, Gottschall and Kram, 2005). These proportions are independent of speed in helmeted guinea fowl (*Numida meleagris*) even though duty factor decreased with speed, which is contradictory to the interspecific comparison (Kram and Taylor, 1990, Taylor et al., 1980). Additionally, the length of the limb has also been shown to influence mass specific GCOT, such that limb length is a more effective parameter to predict GCOT than body mass; the longer the limbs, the lower the mass specific GCOT (Pontzer, 2007). Contradictions in these observations depending on the species under investigation, suggest the presence of some additional complex relationship amongst the parameters

influencing GCOT, and illustrate the ongoing debate about the mechanisms affecting the cost of pedestrian transport.

Body mass is the most widely used controllable parameter to understand the influence that other parameters may have on the cost of transport. Indeed it is the one of the only changeable parameters for experiment within the same individual and within the same gait. Furthermore, the effect of body mass is also a peculiar example. As previously mentioned, heavier species are more efficient at walking, and thus have a lower mass-specific GCOT than lighter species. However, within a species an increase in body mass tends to reduce the efficiency in energy expenditure of transport, as indicated in studies comparing individuals from the same species but at different body mass conditions, or comparing the same individual either bearing an added load or not (Browning et al., 2006, Marsh et al., 2006, Griffin et al., 2003, Tickle et al., 2010, Taylor et al., 1980, Askew et al., 2012). Different load costs have also been demonstrated for the same load (same weight and location e.g. trunk, limbs) but for different gaits (Marsh et al., 2006). Marsh (2006) suggested that the lack of proportional changes in the cost for the same load at different speeds was due to the differing duty factor between walking and running, with walking being more affected by an increase in mass as the stance phase is longer than during running (Marsh et al., 2006). A few species have shown an extraordinary efficiency in energy expenditure during load transport for instance rhinoceros beetles (Scarabaeidae; Kram, 1996), humans (*Homo sapiens sapiens*; Bastien et al., 2005, Maloiy et al., 1986, Heglund et al., 1995), king penguins (*Aptenodytes patagonicus*; Halsey et al., 2007b) Svalbard Rock Ptarmigan (*Lagopus muta hyperborean*; Lees et al., 2010). Investigations on these specific species or ways of carrying loads (e.g. in humans) have attempted to explain this efficiency, but no clear reason has been found.

Research which has split GCOT by describing separately internal and external work, or by stance and swing phases, indicates the importance of partitioning (e.g. Steudel, 1990 demonstrated the influence of load position on the GCOT, as well as, suggesting the importance of swing phase in determining GCOT; Marsh et al., 2004). Using this partitioning approach could help to better understand the parameters influencing GCOT, as, potentially, the same parameter may influence NCOT more than PCOT. For instance, swing phase could be potentially more correlated to the NCOT, while the stance phase could be linked with PCOT, although this hypothesis needs further studies for confirmation. Only a few studies looked at GCOT as a function of NCOT and PCOT (Halsey et al., 2007b, Halsey, 2013), or focused on the parameters influencing specifically NCOT or PCOT (Halsey, 2013). Mass-independent PCOT (without taking upright bird as penguins in consideration) has been shown to be negatively correlated with limb length in birds (Halsey, 2013). NCOT of walking king penguin has been shown to be independent of their body mass (Halsey et al., 2007b). Apart from this, no research studying specifically the parameters influencing only NCOT has been undertaken.

Consequently, the effect of body mass on NCOT independent of PCOT is still unknown. However, from extrapolation of previous research using the relationship $y = a\,x + b$ (Taylor et al., 1970, Roberts et al., 1998, Kram and Taylor, 1990, Marsh et al., 2006, Griffin et al., 2003, Browning et al., 2006, Maloiy et al., 1986), the effect of body mass on NCOT could be demonstrated. The same tendency as for GCOT has been found: an increase in mass-specific NCOT efficiency in energy expenditure in heavier species (Taylor et al., 1970, Roberts et al., 1998, Kram and Taylor, 1990) but a reduction of efficiency with increasing mass within a species (Browning et al., 2006, Marsh et al., 2006, Griffin et al., 2003, Maloiy et al., 1986). Results of load carried as backpacks in army recruits (Goldman and Iampietro, 1962, Pandolf et al., 1977), or of humans with higher body mass condition (Browning et al.,

2006, Griffin et al., 2003), showed an increase in NCOT. Some exceptions have been found in certain ethnic groups, who perform a head-supported load carriage of 20% of their body mass with no additional NCOT. The mechanics behind this optimised cost of carrying load are, however, still unclear (Cavagna et al., 2002, Bastien et al., 2005, Minetti et al., 2006). Halsey (2007b) has shown that king penguins' NCOT was independent of changes in body mass. This means that these birds paradoxically use the same amount of energy per unit distance when heavy (on their way to the zone of attachment) as when light (on their return to the sea). Studies on three other birds species showed a better efficiency in energy expenditure when carrying load than mammals, either by a reduced proportional increase (Tickle et al., 2010, Marsh et al., 2006, McGowan et al., 2006), or by a decrease of energy consumed per loaded mass (Lees et al., 2010). Thus more studies are needed to ascertain whether this aptitude is general to birds. However, understanding the mechanism behind this optimised cost of transport (i.e. unchanging NCOT) could enable a better understanding of the parameters influencing the GCOT.

In the context of king penguin's ecology, this optimisation represents an advantageous evolutionary adaptation (Witter and Cuthill, 1993) to their extreme living context. King penguins have a constrained onshore energy budget and need to walk several kilometres to and from their zone of attachment in both fed and fasted states. They are fasting up to one month and during this period they need to walk several kilometres to reach their zone of attachment and still have enough energy to efficiently fish for prey when back in the ocean (Barrat, 1976). Thus their energy budget management is very important. Furthermore, penguins are known to have a high terrestrial GCOT compared to other species (Pinshow et al., 1976a, Pinshow et al., 1976b, Halsey et al., 2007b, Dewasmes et al., 1980), representing, however, only a small part of their global onshore energy expenditure (Halsey et al., 2007b) which includes, metabolic- , defence-, comfort- costs etc. The skill of optimisation in load

carrying while walking is particularly remarkable given that penguins are primarily swimmers and not walkers. Both constraints (i.e. pedestrian locomotion being their secondary locomotion mode and having to walk with a wide range of body masses) engendered an efficient adaptation in penguins, making their walk ecologically and evolutionarily interesting. Waddling has been suggested as an explanation for the high cost of penguin pedestrian locomotion. However, good energy transfer within each waddle has been demonstrated (Griffin and Kram, 2000), suggesting that the high energy cost results from their relatively short leg length. This requires penguins to generate muscular force more rapidly resulting in a higher number of strides per unit distance relative to another animal of the same body mass (Griffin and Kram, 2000). This represents a morphological trade-off for king penguins between swimming and walking energy efficiency. Their hydrodynamic shape includes short legs placed in line with their body (with the use of flippers for propulsion), resulting in an upright posture during pedestrian locomotion.

Therefore to try to better understand the parameters that influence GCOT, this study looked at the energetics and the biomechanics of pedestrian locomotion in king penguins, by attempting to understand the mechanism behind this optimisation of their mass independent NCOT, using body mass change as variable parameter. The energy measurement protocol from Halsey et al. (2007b) was applied on 10 birds, while biomechanical measures were recorded during four treadmill sessions involving four different speeds. The study was structured with three aims: (1) The first aim was to determine if the effect of mass on the energy expenditure in the data collected in this present conformed with previous research (Halsey et al., 2007b), which showed that NCOT was independent from body mass (2) The second aim was to determine if any changes in temporal and spatial characteristics of penguin gait with body mass could be an explanatory mechanism. Tri-axial acceleration measurements (expressed as Vectorial Dynamic Body Acceleration, VeDBA) along with

three dimensional reconstruction of walking gait were used to quantify the gait patterns, and changes in the following parameters were chosen for analysis: global change of gait, stride frequency, stride length, duty factor, stride width, stability and change of position of the centre of mass. Finally (3) the third aim was to determine if the location of the centre of mass of a light and a heavy penguin could be a parameter explaining any alterations in gait and thus the optimised cost of load carrying, i.e. 'the optimised fat penguin'. We hypothesised the following parameters to decrease progressively with decreasing body mass as a result of fasting: step width, stride frequency, stance duration, roll and pitch. Further, we hypothes§ised that the stride length, stride duration and swing duration would increase with decreasing body mass. These adaptations would result in a greater energetic efficiency during pedestrian locomotion by heavier king penguins. Finally we hypothesised to find a backward translation of the center of mass position with decreasing body mass.

5.3 Materials and methods

5.3.1 Birds and experimental protocol

5.3.1.1 Biomechanics and energy expenditure of walking king penguins

Penguins were captured in the morning and soon afterwards their ability to walk on a treadmill was assessed. When enough penguins suitable for the treadmill had been captured, data collection began. The first experiment generally took place on the same day or the day following capture. The penguins of group D were kept in a pen until the end of experiments (Table 2-1). Before the experiment, each bird was weighed and equipped with the two data loggers (heart rate data logger § 2.1.6, and triaxial acceleration data logger § 2.1.7.). The bird was then placed in the respirometer chamber upon a treadmill such that he walked at controlled speeds. The $\dot{V}_{O_2}$ and VeDBA of courting birds were measured as soon as the bird was put in the respirometer chamber. The bird rested for one hour before the treadmill was turned on, thus requiring the bird to walk (Figure 2.20 for an example of the experiment schedule). Then, an initial walking session of five minutes was completed to acclimate the

bird to walking on the treadmill. The experiment involved four walking sessions at speeds of 1, 1.2, 1.4 and 1.6 km/h, with 10 minutes rest between each on a flat surface. Experiments and data collection were repeated four times at approximately days 0, 7, 14 and 21, with the respective average body masses referred to as 'heaviest' (13.2 kg), 'heavy'(11.7 kg), 'light'(11.0 kg) and 'lightest' (9.8 kg). Birds were kept in a pen after the experiment and released at the same place in the colony after the fourth experiment.

5.3.1.2 Location of the centre of mass

The location of the centre of mass was determined using the multiple suspension method (Abourachid, 1993), on the body of both acquired king penguin cadavers (individuals of groups E, Table 2-1). In a rigid body, the centre of mass can be determined by suspension (with a rope, for example) of the body from different places on the body. The rope axis will always intersect the centre of mass of a stabilised, suspended body that is free of movement. Photoshop (Adobe Elements 6.0) or Inkscape (Inkscape 0.48, www.inkscape.org) software were used to visually determine the centre of mass from photography of cadaver suspension.

5.3.2 Data processing and statistical analysis

5.3.2.1 Energetics: Is NCOT mass independent?

As demonstrated (§ 2.1.2.1), $\dot{V}_{O_2}$ in king penguins needed almost one minute to react. Thus, the best representation of the stabilised $\dot{V}_{O_2}$ was a mean of the entire walking session excluding the first minute. To test the effect of Mass on NCOT, repeated ANCOVA of the mixed model $\dot{V}_{O_2}$= Speed* Mass+ Mass+ Individual[random] was completed with the package 'lme4' from R Cran (R Core Team, 2012) (unbalanced N = 10, 10, 8, 10, for heaviest, heavy, light and lightest, respectively). King penguins did not have a fluid walk at the lowest speeds encountered in previous studies (Halsey et al., 2007b, Fahlman et al., 2004), thus, as the biomechanics data were collected simultaneously with the energetic data, the used range speed was from 1.0 to 1.6 km/h.

5.3.2.2 Biomechanics: How does the gait adapt along with body mass?

Stride

Biomechanical data were collected from two synchronised videos at different angles (50 Hz for 15 sec) while a king penguin was walking at 1.4 km/h (the modal speed within the breeding colony; pers. ob.). These recordings included a minimum of 10 stride cycles. See chapter two on 'General Methods' (§ 2.1.8) for further information about the material and its calibration. Stride parameters (length, frequency and duration, as well as left to right step width) were geometrically calculated from the three dimensional coordinates of left feet heel at initial contact. Stance duration, swing duration and duty factor were calculated with the initial contact and the toe-off of the left feet. Data were repeated measures of four birds and four body masses.

Means and standard deviations of the stride parameters (i.e. length, frequency, duration, stance duration, swing duration and duty factor) and step width were calculated for each individual at each body mass from the videos (N = 4,3,2,4, for heaviest, heavy, light and lightest body masses, respectively). Additionally, stride frequency was calculated from the dynamic body acceleration of the Z (vertical) axis. The maxima of the graphical representation of vertical Z-axis (i.e. DBA_z) are linked to the highest acceleration of the stride cycle, which is presumably occurring at the heel strike (Figure 5.2). One maximum can be thus associated with a heel strike. The first minute data of the 10-minute walking session were removed as some birds presented an irregular walk at the start of the experiment. The mean and standard deviation from every two maxima (i.e. steps frequency) of the last nine minutes were calculated per individual, while walking at 1.4 km/h and for each body condition (N=8, 8, 10, 6, for heaviest, heavy, light and lightest body condition, respectively). Due to the small sample size for the video-derived data descriptive analyses were completed, however statistical analyses were also completed to indicate where

statistical differences or tendencies were found. Linear design with individual as random factor was used with the package 'lme4' from R Cran (R Core Team, 2012): Strides/Step parameter = Mass + Individual [random]. Repeated ANCOVA of the mixed model was completed to test the effect of body mass for each parameter (N=8, 8, 10, 6, for heaviest, heavy, light and lightest body condition, respectively). Standard deviations were used as a measure of parameter repeatability. Parameter mean or standard deviation having a significant P value were further analysed with post hoc Wilcoxon signed-ranks tests and paired t-tests, with P value adjusted by Holm, Bonferroni or without adjustment method. However only p-value from the t-test are shown in this chapter to avoid confusion. Global mean of individual means and standard deviations were calculated for illustration purpose of Table 5-3.

Comparison of data taken from the videos versus data taken from the accelerometer
Due to the small amount of data obtained from video footage (N = 4,3,2,4, for heaviest, heavy, light and lightest masses, respectively), the conformity of results of data taken from the videos versus data taken from the accelerometer was tested. To do so comparisons on the means and standard deviation of stride frequency of data from the two different methods (N=8, 8, 10, 6, for heaviest, heavy, light and lightest body condition, respectively) were conducted by paired t-test of each body mass condition.

Gait
Mean VeDBA was calculated for the last nine minutes of walking per individual (§ 2.3.1.1), as some birds presented an irregular walk at the start of the experiment. A repeated ANCOVA of the mixed model VeDBA = Speed * Mass + Mass + Individual [random] was done to test the change in VeDBA along with change of body mass (N = 10, 10, 8, 10, for heaviest, heavy, light and lightest body condition, respectively).

The frequency of peaks (number of peaks -minimum and maximum- per minute) and the amplitude (i.e. between a minimum and its next maximum) of each DBA were calculated using a custom-written script in MATLAB by Yves Handrich (MATLAB, 2010) using an algorithm to ignore sub-peaks due to noise (Figure 5.2). Data from the first minute were removed to let the bird reach a fluent gait. Mean and standard deviation of the nine last minutes were calculated for each parameter per individual at each body mass condition. Repeated measured ANCOVA of the linear mixed model Gait parameter = Mass+ Individual[random] were done to test the effect of body mass on the individual mean and standard deviation of peaks frequency and their amplitude (N=8, 8, 10, 6, for heaviest, heavy, light and lightest body condition, respectively) (package 'lme4' R Core Team, 2012). Global means of individual means for each parameter were made for illustration purpose of Table 5-3.

To assess the extent to which the bird waddled and tilted, the roll and pitch amplitudes of the oscillation angle around the static body acceleration (SBA) of X and Y axes (i.e. roll and pitch, respectively) were calculated. A custom-written script by Yves Handrich in MATLAB (MATLAB, 2010) was made to find not more than one minimum and one maximum angle of roll in each stride (i.e. between three following maxima of DBA_z, see Figure 5.3) and to find not more than one minimum and one maximum angles of pitch in each step (i.e. between two following maxima of DBA_z, see Figure 5.3). As for DBA, the mean and standard deviation of the last nine minutes of data were calculated per individual at each body mass condition. The effect of body mass was tested on each data with a repeated measured ANCOVA of the linear mixed model Amplitude = Mass+ Individual[random] (N=8, 8, 10, 6, for heaviest, heavy, light and lightest body condition, respectively). Global mean of individual means and standard deviation were made for illustration purpose of Table 5-6.

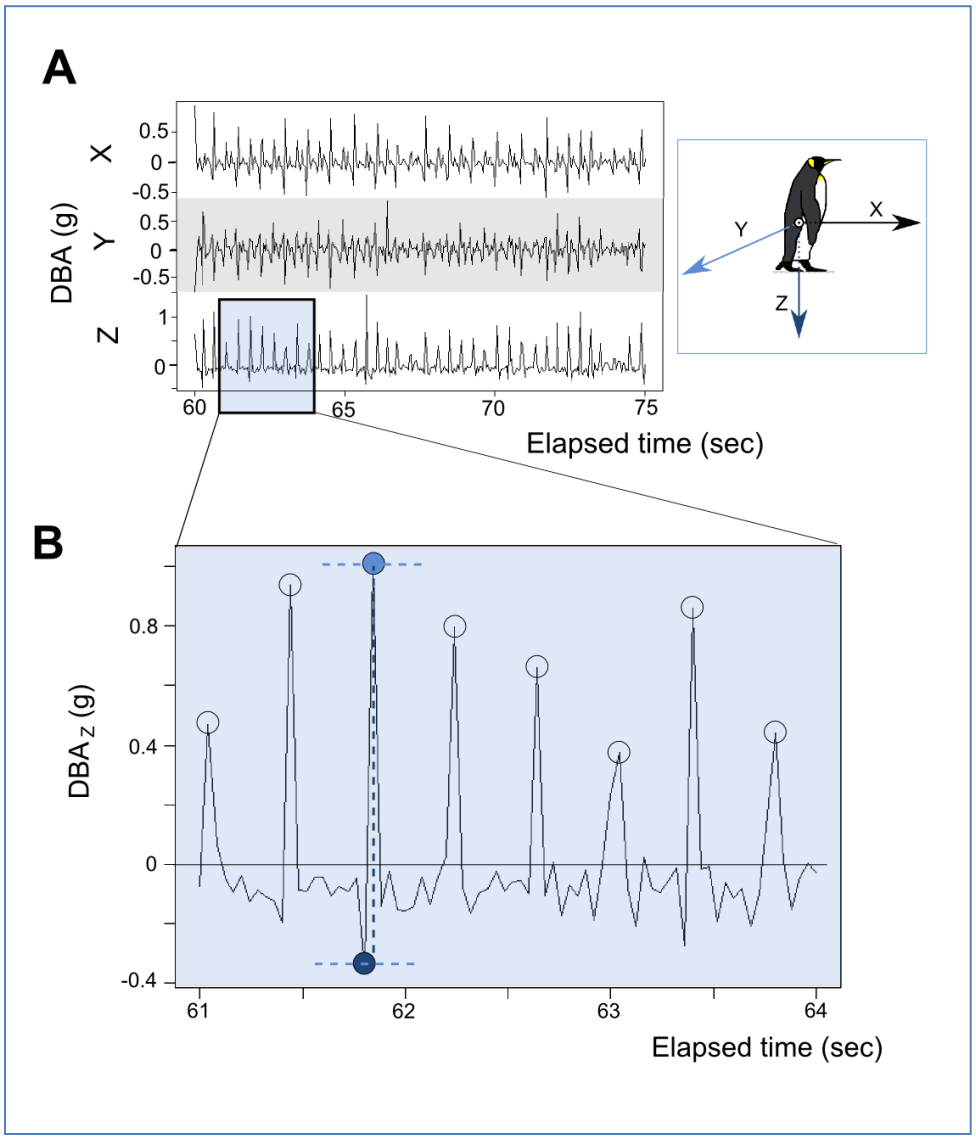


Figure 5.2 A. Left: Example of a graphical representation of three axes of the dynamic body acceleration (DBA) data within 15 seconds. Right: Visual representation of the DBA axes. B. Zoom in the DBAz. Black circles represent all peaks considered as maxima by Yves Handrich's algorithm. The sub-peaks are ignored thanks to this algorithm. These maxima represent the heel strike of the king penguin. The dark blue circles represent minima, while the light blue one represents next maxima. The horizontal dashed lines represent the height of the maxima/minima, while the vertical dashed line represents the amplitudes between the minimum and its next maximum peaks.

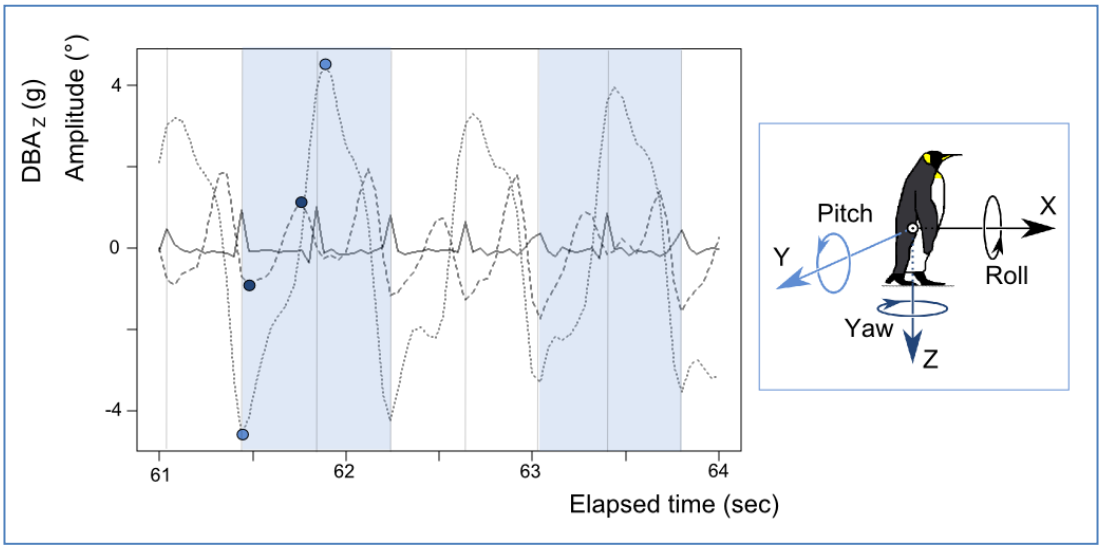


Figure 5.3 Left: Example of a graphical representation of the dynamic body acceleration of Z axis (DBAz; plain line), roll (dotted line) and of the pitch (dashed line) in function of the time. The successive light blue and white shades represent one 'stride' starting at heel strike of the left feet. The grey verticals represent separation between two 'steps'. One maximum and one minimum of pitch within one 'step' are represented in dark blue circles, while one maximum and one minimum of roll within one 'stride' are represented in light blue circles. **Right: Visual representation of the DBA axes and angle movements.**

Centre of mass

Visual comparison of the localisation of the centre of mass was made using Inkscape (Inkscape 0.48, www.inkscape.org) software.

Table 5-1 Analyses summary; A=accelerometry data, V= videos data.

Aims						Birds type	Statistical analysis	Variables
Energetic	**Is NCOT mass independent?**	***Effect of body mass on partitioned GCOT*** ***GCOT =NCOT*Speed + PCOT***				Ten bird in courtship (group D)	Repeated ANCOVA of the linear mixed model	$\dot{V}_{O_2}$ = Speed* Mass+ Mass+ Individual [random]. Mean of $\dot{V}_{O_2}$ collected during of each speed-specific walking sessions per individual.
Biomechanics	**How does the gait adapt along with body mass?**	***Stride and it repeata bility***	***Length***				Descriptive analysis as well as repeated ANCOVA of the linear mixed model Post hoc: paired t-test with P adjusted by Holm, Bonferroni or not.	Stride/Step parameter = Mass +Individual [random]. Mean of each parameters and their standard deviations per individual at each mass condition while walking at 1.4km/h.
			Step width					
			Frequency					
			Duration					
			Stand Duration					
			Swing Duration					
			Duty factor					
							t-test of stride frequency	*Mean of stride frequency taken from accelerometer or video per mass condition. The four same individuals' data.*
		Gait and its repeata bility	***Global Gait (VeDBA)***				Repeated ANCOVA of the mixed model	VeDBA = Speed+ Mass+ Individual [random]. Mean of VeDBA collected during each speed-specific walking sessions per individual.
			BA	***Dyn.***	***DBA_X***		Repeated measures ANCOVA of the mixed model	Gait parameter = Mass + Individual [random]. Mean and standard deviation, per individual at each mass condition, of the peaks frequency and the amplitude between two min/max peaks, while walking at 1.4km/h.
					DBA_Y			
					DBA_Z			
				Static Posture	**Roll (SBA_X)**		Repeated measures ANCOVA of the mixed model	Amplitude = Mass + Individual [random]. Mean and standard deviation, per individual at each mass condition, of the amplitude between two min/max peaks, while walking at 1.4km/h.
					Pitch (SBA_y)			
	Location of centre of mass and its change					Two dead birds (group E).	NA	Pictures of suspension of body with different body mass conditions.

5.4 Results

See Appendices for raw data.

5.4.1 Energetics: Is NCOT mass independent?

Repeated ANCOVA showed a significant positive effect of mass on the intercept of the model ($P < 0.001$, $N=10$), while no effect was found on the slope ($P= 0.515$, $N=10$, Table 5-2 and Figure 5.4).

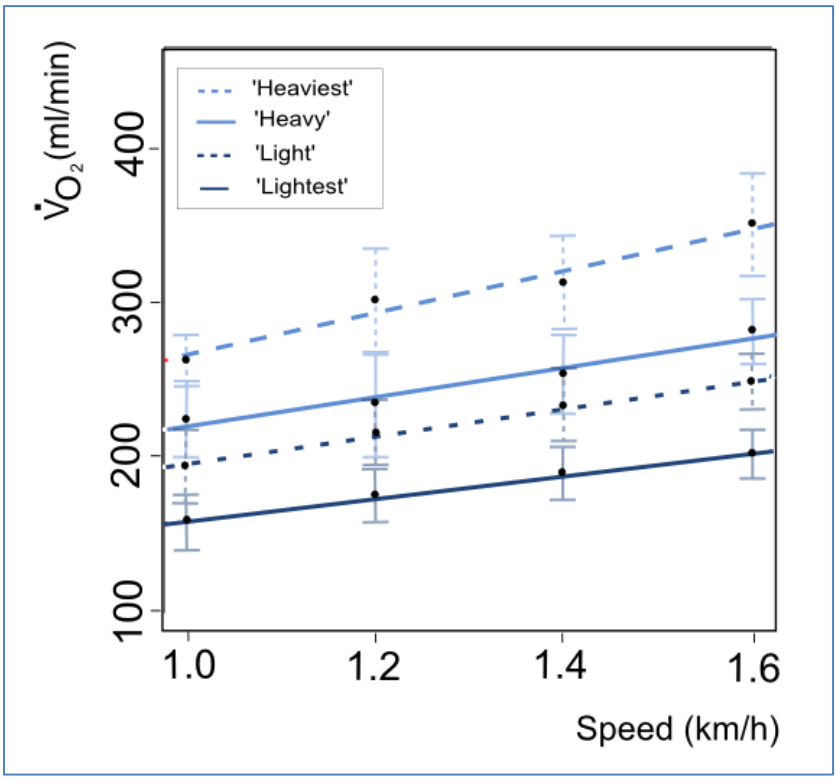


Figure 5.4 Graphical representation of the effect of mass on GCOT (GCOT =NCOT*Speed + PCOT); where GCOT is represented as $\dot{V}_{O_2}$. The plain dark line is the GCOT for the "lightest" mass, the dark dashed line for "light", the plain light line is for "heavy" and the dashed light line is for the "heaviest" mass. The black dots are the measured data, while whiskers represent ± 1 SD.

Table 5-2 Effect of mass on GCOT (GCOT =NCOT*Speed + PCOT): ANCOVA Table of linear mixed-effect model $\dot{V}_{O_2}$= Speed* Mass+ Mass+ individual [random]. The asterisk represents P <0.05.

Variables	DF	Sum Sq	Mean Sq	F value	P value
Speed	3	41584	13861	39.391	$< 2x\ 10^{-16}$*
Mass	3	142328	47443	134.824	$< 2x\ 10^{-16}$*
Speed* Mass	9	3013	335	0.951	0.515

5.4.2 Biomechanics: How does gait related to body mass?

5.4.2.1 Stride parameters

The results of the repeated ANCOVA indicated no effect of body mass on any of the stride parameters except for the step width (P = 0.0143, N = 4,3,2,4, for heaviest, heavy, light and lightest body condition, respectively; see Table 5-3). Post-hoc pairwise t-test showed no significant differences between body masses, however P = 0.06 when comparing the lightest and heaviest body masses. Descriptively, step width slightly increased within the fasting period to finally reduce at the lightest body mass. This pattern was similar when repeatability of step width was assessed (Table 5-3). Repeatability (i.e. repeated ANCOVA the standard deviation) within each parameter is not significantly different, except for step width (P = 0.001, N = 4,3,2,4, for heaviest, heavy, light and lightest body condition, respectively, Table 5-3). Post-hoc analyses showed that the significantly different pairs are between the data for heavy-lightest and light-lightest (P value = 0.018, and 0.015, respectively. N = 4,3,2,4, for heaviest, heavy, light and lightest body condition, respectively), when the P value was not adjusted. When the P value was adjusted for multiple comparisons (Holm or Bonferroni method), no differences were found (N = 4,3,2,4, for heaviest, heavy, light and lightest body condition, respectively). Even though statistical analyse are not significantly different, the standard deviation of the strides had a trend toward frequency decreasing with a decrease of body mass, as well as the standard deviation of the stride duration.

Data taken from the videos versus data taken from the accelerometer

No differences in the stride frequency were found between the data from the accelerometer and from the digitised data using a t-test of each body mass condition using the same individuals (P = 0.878, N = 4; P = 0.6, N = 3; P = 0.95, N = 2; P = 0.618, N = 4; for heaviest, heavy, light and the lightest body mass conditions, respectively). Therefore it was felt that the accelerometer data could be used to quantify stride frequency over a longer period and for more birds.

Table 5-3 Table of means of strides parameters while walking at 1.4 km/hour, along with different body mass condition. The asterisks represent P value <0.05. V= Video data; A= Accelerometry data.

Parameters mean of stride		Body Mass				P value	Source
		Heaviest (~13.2 kg)	Heavy (~11.7 kg)	Light (~11.0 kg)	Lightest (~9.8 kg)		
Length [m]	Mean	0.349	0.338	0.345	0.338	0.686	V
	SD	0.031	0.029	0.034	0.026	0.125	
Step width [m]	Mean	0.106	0.108	0.118	0.089	0.0143*	V
	SD	0.017	0.023	0.024	0.014	0.001*	
Frequency [s^{-1}]	Mean	1.303	1.332	1.296	1.290	0.851	V
	SD	0.112	0.108	0.090	0.085	0.391	
	Mean	1.304	1.273	1.252	1.272	0.372	A
	SD	0.191	0.172	0.139	0.155	0.784	
Duration [s]	Mean	0.777	0.759	0.781	0.779	0.863	V
	SD	0.062	0.059	0.057	0.051	0.360	
Stance duration [s]	Mean	0.522	0.503	0.518	0.518	0.835	V
	SD	0.055	0.050	0.059	0.045	0.078	
Swing duration [s]	Mean	0.253	0.256	0.259	0.261	0.706	V
	SD	0.029	0.030	0.022	0.028	0.208	
Duty Factor [%]	Mean	67.195	66.347	66.294	66.467	0.318	V
	SD	0.035	0.033	0.034	0.033	0.942	

5.4.2.2 Global change of gait

5.4.2.2.1 VeDBA

Repeated measures ANCOVA of the mixed model showed a significant positive effect of the mass on the intercept (P > 0.001), while no effect was found on the slope (P= 0.747, Table 5-4). N = 10, 10, 8, 10, for heaviest, heavy, light and lightest body condition, respectively.

Table 5-4 Table of the effect of masse: repeated measures ANCOVA for linear mixed-effect model of VeDBA in function of the speed. The asterisk represents P value <0.05

Variables	DF	Sum Sq	Mean Sq	F value	P value
Speed	3	18.619	6.207	105.263	$< 2x\ 10^{-16}$*
Mass	3	5.062	1.687	28.619	1.99×10^{-12}*
Speed* Mass	9	0.362	0.040	0.681	0.747

5.4.2.2.2 DBA

The results of the repeated measures ANCOVA of the model indicated no significant effect of body mass on any of the DBA either for peak frequency or amplitude (see Table 5-5. N=8, 8, 10, 6, for heaviest, heavy, light and lightest body condition, respectively).

Table 5-5 Mean of peaks number and the mean amplitude of peaks for each DBA while walking at 1.4 km/hour, along with different body mass conditions. The asterisks represent P value <0.05

	Parameter mean	Body Mass				P value
		Heaviest (~13.2 kg)	Heavy (~11.7 kg)	Light (~11.0 kg)	Lightest (~9.8 kg)	
DBA_X	Peaks frequency [min^{-1}]	154.326	149.938	143.178	150.987	0.455
	Amplitude [ms^{-2}]	0.446	0.469	0.439	0.463	0.847
DBA_Y	Peaks frequency [min^{-1}]	194.812	200.948	192.322	194.821	0.898
	Amplitude [ms^{-2}]	0.676	0.636	0.596	0.583	0.065
DBA_Z	Peaks frequency [min^{-1}]	150.276	148.947	143.078	149.551	0.661
	Amplitude [ms^{-2}]	0.741	0.735	0.677	0.761	0.497

5.4.2.3 Static, posture: Roll and pitch

Table 5-6 Table of the angle amplitude from the posture while walking at 1.4 km/hour. The asterisks represent P value <0.05.

Parameter mean		Body Mass				P value
		Heaviest (~13.2 kg)	Heavy (~11.7 kg)	Light (~11.0 kg)	Lightest (~9.8 kg)	
Roll amplitude [°]	Mean	9.025	8.931	7.209	8.955	0.045*
	SD	1.828	1.965	1.202	1.679	0.341
Pitch amplitude [°]	Mean	2.215	2.369	2.005	2.795	0.010*
	SD	0.966	0.823	0.648	1.046	0.331

Repeated measures ANCOVA of the model on the roll (waddle) and the pitch (tilt) showed a significant effect of body mass on both movements (P = 0.045 and 0.010, for roll and pitch respectively, N=8, 8, 10, 6, for heaviest, heavy, light and lightest body condition, respectively). Post hoc Wilcoxon pairwise comparisons with the adjustment of P value using Holm or Bonferroni method did not show any significant differences for the roll. Only the paired comparisons for heaviest-light and heavy-light were significantly different (P= 0.03 and 0.04, N= 8 and 10, respectively) when the P value was not adjusted to the multiple comparisons of roll. None of the post hoc tests showed any significant differences between different body masses on the pitch. However probably the underlying differences occurred

between heaviest-light and heavy-light (P=0.08, 0.09, respectively, with no P value adjustment). Repeatability along with change of body mass did not show any significant difference (Table 5-6).

5.4.3 Biomechanics: The centre of mass

The heavy king penguin cadaver was preserved in alcohol, which slowly penetrated the body. As the organs of the bird started to ferment, the body had to be opened to release the formed gas. Additionally, the lack of anatomical markers before preservation made it impossible to perform a good comparison of both bodies. The posture of the heavy cadaver was not akin to a natural walking posture, due to the cramped confines of the container within which the cadaver was stored. The light cadaver was maintained in a similar posture to the heavy cadaver (frozen) to aid comparison. See Figure 5.5 and Figure 5.6 for an overview of the attempts to measure the centre of mass for the two cadavers.

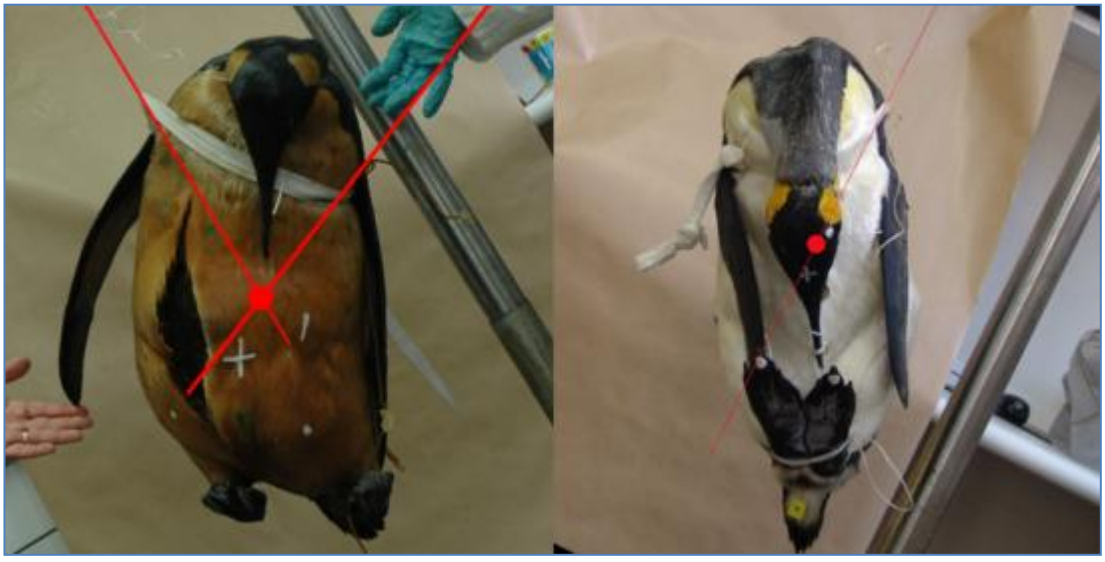


Figure 5.5 Frontal location of the centre of mass. Left: heavy cadaver, right: light cadaver. Lines were drawn to find the centre of mass from the suspension method.

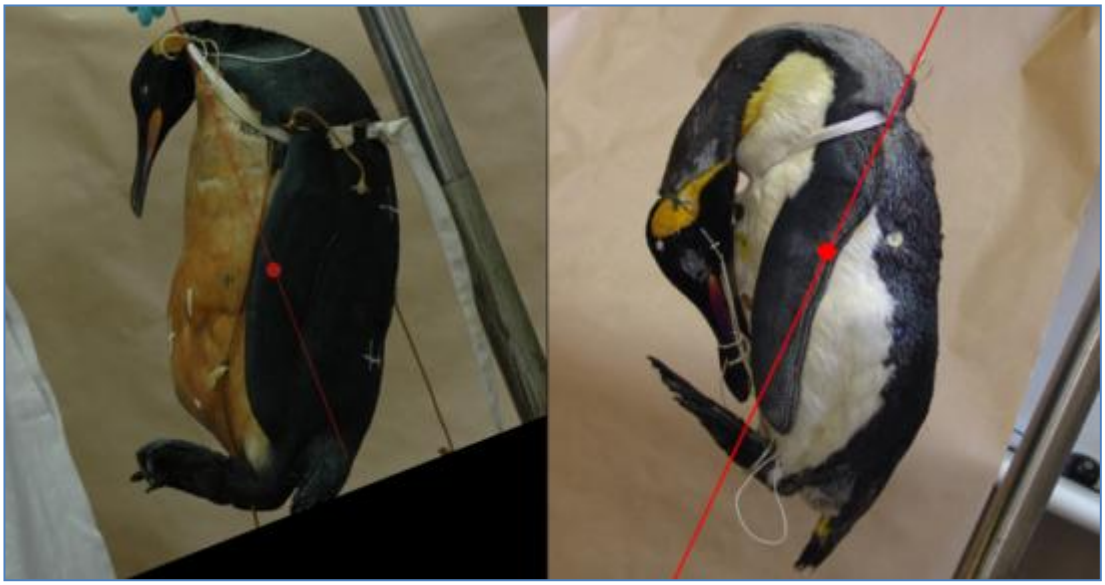


Figure 5.6 Lateral location of the centre of mass. Left: heavy cadaver, right: light cadaver. Lines were drawn to find the centre of mass from the suspension method.

Although an imprecise comparison, the results showed no evidence for a difference in location of the centre of mass between heavier and lighter king penguins.

5.5 Discussion

The energy expenditure data measured in the present study were consistent with previously reported data (Halsey et al. 2007) in showing that the relationship between $\dot{V}_{O_2}$ and speed (NCOT) is independent of mass in king penguins, supporting the hypothesis of the optimised fat king penguins.

In terms of uncovering the parameters influencing the cost of transport, analyses of the accelerations did not show any changes for any of the axes, although the global tri-axial acceleration of the body was shown to be dependent on mass. Consequently, king penguins kept the same gait accelerations when walking with different body mass, attesting that mass independency of NCOT was not linked with a change of gait.

The cost of gait has been primarly defined by the cost of supporting body mass during the stance phase related to the time of the stance phase (Kram and Taylor, 1990). Studies analysing the effect of carrying a load have found that loaded horses increase the stance phase by 19% when compared to the unloaded condition (Hoyt et al., 2000), which has been used to explain the increase of energy expenditure. A similar finding has been seen in bipedal animals (Marsh et al., 2006, Griffin et al., 2003). However, other studies found no biomechanical changes in stance timing while loaded (Taylor et al., 1980, Tickle et al., 2010, McGowan et al., 2006). The results of this research showed that king penguins do not decrease their stance time, stride length or stride frequency when heavier.

Swing phase duration has also been found to have an impact on the GCOT (Marsh et al., 2004, Modica and Kram, 2005, Doke et al., 2005, Gottschall and Kram, 2005), thus a change in its duration could explain a change of NCOT. Nonetheless these present results did not show any changes in the swing phase duration, or in the duty factor along with the change of body mass.

An additional theory could be that increased stability in movement, indicated through greater repeatibility of the cyclic pattern is likely to result in reducing energy expenditure of locomotion. Conversely, a variable gait pattern will require many adaptations, over a number of steps to maintain locomotion, which, in theory, would result in more energy consumption. The current results indcated that none of the parameters related to regularity were statistically different as body mass changed. However, the repetability of step width tended to decrease with the decrease of bodymass. This tends to suggest that the hypothesis that there will be a reduction in cost with increase in variability for heavy king penguin may be true, though more research is required to explore this further.

In the stride parameters, only the step width has been demonstrated to have a significant relationship with body mass. The dynamic gait theory suggests that step width has a greater influence than step length on the cost of walking. Increase in width has been shown to mathematically increase the mechanical work on the centre of mass and consequently on the metabolic rate, to the power of four while an increase of stride length to the power of two (Kuo et al., 2005). The width slightly increased within the fasting period but reduced at the lightest body mass. And this slight increase in width during the fasting period of penguins may lead to an increase of energy expenditure, at least until the light condition. This may explain the optimisation of heavy king penguins in NCOT. The present study, in contrast to Kurz et al. (2008), does not show that king penguin have higher stability in step width than stride length (Kurz et al., 2008). However, the standard deviation (variability) about the mean step width also increased, to again reduce for the lightest body mass. This is in accordance with the energetic optimisation, showing that heavy king penguins are as efficient as light ones on the NCOT, at least until the light condition (11.0 kg).

The roll amplitude (waddling) tended to decrease with body mass until the lightest body mass when an increase occurred, in contrast to the width and its variability, which were increasing within the fasting period to finally reduce at the lightest body mass. Waddling has been demonstrated to have a good energy transfer, which enables this movement to be less energy consuming than it looks (Griffin and Kram, 2000), thus a change in waddling should not affect the NCOT. Finally pitch has been shown to generally decrease with body mass, representing an increase in the frontward-backward movements while in the lightest body mass condition, which probably demands an increase in energy.

As previously mentioned, studies involving head-supported load demonstrate extra load carrying with less or no additional NCOT (Maloiy et al., 1986, Minetti et al., 2006, Bastien

et al., 2005), while army recruits carrying the same weight as a backpack showed an increase of the NCOT (Pandolf et al., 1977, Goldman and Iampietro, 1962). Those studies showed that energy expenditure while walking with a load depend principally on how the load is carried, suggesting (without proving) that the change in height, and horizontal distance of the centre of mass from the base of support explains the change in energy expenditure. However training in this method is needed as untrained Europeans were not efficient in head load carrying. The process of this optimisation was examined further (Heglund et al., 1995, Cavagna et al., 2002), demonstrating that loading in African women significantly improved the transduction of potential to kinetic energy during the descent of the centre of mass, while the change in European ethnic group while using the same carrying method was not significant. Further data collection and biomechanical analysis are required to determine the mechanical processes behind this phenomenon and thus far the authors of this research have been unable to propose a viable explanation. Unfortunately, the location of the centre of mass in king penguins and its change with different body mass conditions was not successful.

In summary the only biomechanical parameters which showed a significant difference that could be used to explain the optimisation of energy expenditure by fat penguins were step width and its stability. Waddling and pitch are significantly changing. However, waddling should not affect the GCOT, while no research on the effect of pitch on GCOT has been found.

However, a potential interpretation can be suggested along with the previously mentioned results. Potentially, the centre of mass of a heavy and a light king penguin change slightly in the sagittal plan, i.e. moving frontward when the bird is heavier. The reduction in abdominal fat accumulation has been demonstrated to first decrease abruptly at the start of the fasting,

while the subcutaneous fat accumulation decreased constantly but progressively along the fasting period in emperor penguins (Dewasmes et al., 1980). This phenomenon was also observed in king penguins (personal observations), where heavy birds have a bigger abdomen in proportion to lighter ones. The consequent anterior location of the centre of mass, relative to the feet will likely aid progression and efficiency, as the momentum from the forward fall will advance the gait without the need for a large push-off contribution from the muscles. Potentially more energy will be required to control the gait than when the centre of mass was located closer to the feet in the sagittal plane, to avoid too much momentum causing a forward fall, i.e. representing, in absolute terms, the cost of the walking posture and its PCOT (heaviest penguins have a smaller pitch than lightest). It would appear that this mechanism may be more energy efficient overall. Indeed penguin pedestrian locomotion has been shown to be high in energy consumption due to the force generated to move their short limbs (Griffin and Kram, 2000), and not to control the fall. See Figure 5.7 for illustration of the interpretation.

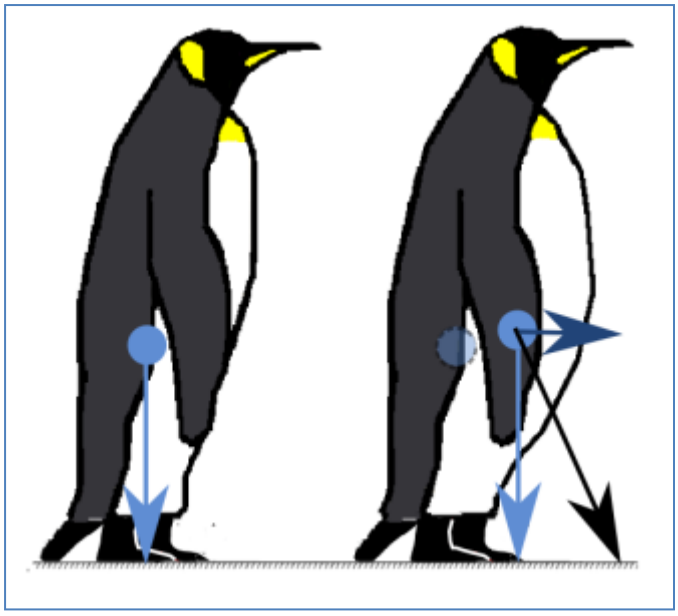


Figure 5.7 Hypothetical change in the location of the centre of mass between light (left) and heavy (right) standing king penguins, generating a new resultant force which leads the birds to fall ahead.

Additional research needs to be done to demonstrate that the independent propriety of NCOT to body mass as exceptional or general for the avian species, and the comparison with another upright bipedal (i.e. human) is interesting. The comparison of heavy human gait to penguin gait is not straightforward, due to the different anthropometric properties of the two species. When normalised to height, the king penguin has a relatively lower centre of mass, the consequence of which is improved stability. Furthermore heavy humans have been shown to have an accumulation of fat between their thighs, limiting their movements (Browning et al., 2006), while penguins did not show such an accumulation. Thus, it would be interesting to see the NCOT of humans carrying a frontal artificial load.

Even though the mechanism of this efficient skill is still unclear, this study showed potential directions for further research, as well as offering a step forward in trying to uncover the parameter influencing GCOT by its partition. Indeed looking at the effect of each parameter specifically on the PCOT or NCOT could enable a better understanding of GCOT. The low number of birds to provide data for analysis from the video could be improved, as well as the location of the centre of mass with new subjects or with CT-scan analyses or the actual birds' bodies. Finally, this study showed the optimisation of the evolutionary adaptation of king penguins' secondary locomotion to enable a good management of their energy budget while walking at a wide scale range of body masses.

6. It costs to be late: investigating the onshore energy expenditure of incubating king penguins.

Estimating the energy expenditure of the longest fasting period of early and late king penguin breeders, developing beyond previous factors by including the energy expenditure due to stressed state and walking on inclines to obtain altitude.

6.1 Abstract

Early and late breeders are known to have differing reproductive success (38.5 versus 21.5%). Although some studies have investigated this difference, none have looked at differences in energy expenditure, due, for instance, to the difference in density of the colony at different times of the year and the consequent increase in aggressive behaviours between nesting adults, or to the longer journey typically required of a late breeder to reach its zone of attachment. The energy expenditure of individual behaviours (such as comfort –preeing, stretching-, defence) has been estimated for incubating birds, as well as the energy expenditure of pedestrian locomotion. However, extra costs incurred due to stress response *per se* or by walking on different inclines has not yet been considered. In this chapter a simple estimate comparing the energy expenditure of the early and late incubating males during the first and second shift (the longest fasting periods) was developed, using previously published energetics data and further such data collected in the laboratory, such as the cost of stress response and walking on an incline. The results indicated that late breeders encounter an energetic disadvantage of a 66% increase compared to early breeders. However, the disadvantage for late breeders of greater walking distances to and from their zone of attachment in the colony represented only a difference of 1.8 % over the 20 days ashore. Furthermore, difference in the choice of the colony for incubating only represent an advantage of 1.8% of a 20-days energy expenditure of a late breeder. These results suggested that the location of the zone of attachment, within or between colonies, is not an important factor influencing the onshore energy expenditure of an incubating male. However, being an early breeder overall provides an energetic advantage representing eight days of extra fasting time between the first and second shift.

6.2 Introduction

An important influence on the reproductive success of the king penguin is arrival time at the colony to start the breeding cycle, epitomised by the higher reproductive success of ‘early breeders’ than ‘late breeders’ (38.5% of the early birds returning to the colony of Baie du

Marin successfully raised a chick in 1998-2000 versus 21.5% of the late breeders, Descamps et al., 2002). In terms of advantages encountered while being onshore, this higher success of early breeder may be linked with, at least, three factors: temporal advantage for chicks, lower density of the colony and better choice of locations for a breeding pair's zone of attachment.

Probably the most important factor of these is the greater time for chicks to reach the minimal body mass (10-12kg) that enables them to survive through the Sub Antarctic winter (Cherel and Lemaho, 1985, Weimerskirch et al., 1992, Van Heezik et al., 1994).

King penguins are highly territorial, and an average rate of 100 interactions/birds/hour has been observed within colonies (Côté, 2000). As the zones of attachment of king penguins are not physically limited or visually defined, breeders aggressively defend this territory. Thus while brooding, birds are subject to frequent acts of aggression from their neighbours. The number of immediate neighbours has been shown to increase the number of aggressive interactions between birds (Côté, 2000), which unsurprisingly, and as confirmed by Viblanc et al. (*in press*), has an extra cost (e.g. 171 kJ/h for aggressive behaviour with physical contact, estimated from heart rate data). The energy expenditure of an adult breeder increases with the population size of the colony, which is probably due to the decrease in the distance between incubating birds (Viblanc et al., *in press*). The number of king penguins in a colony increases over the course of the breeding season until reaching a plateau around mid-January (Viblanc et al., *in press*), suggesting an energetic advantage for early breeders. Thus an increase in aggressive interactions through the breeding season may also lead to a difference in energy expenditure between early and late breeders. In addition, the number of neighbours has been shown to be positively related to cortisone levels (a type of glucocorticoid, which is a stress response hormone) (Viblanc et al., *in review*), suggesting greater levels of stressed state in more densely populated colonies.

Location in the colony may also have an effect on the reproductive success of king penguins. Early breeders tend to have a more central position in the colony (80% of the central birds are early breeders; Côté, 2000), while late breeders are typically relegated to locations further away and/or to peripheral positions (Pers. Obs. / 69% of peripheral birds are late breeders; Côté, 2000). The topographic location of the zone of attachment has important implications for reproductive success, as more distant locations to rivers and the sea are less subjected to flooding (Pers. obs). A location further from the sea obviously increases walking energy expenditure for king penguins, while nests towards the periphery of the colony are twice as likely to be subjected to predation compared to those in a central position (Côté, 2000). In terms of energetic disadvantages, greater walking distances, greater heights to climb or crossing more breeding areas will all result in higher energy expenditure during pedestrian locomotion. As king penguin adults need to walk from the sea to their zone of attachment, or return, typically ten times through the incubating season, a small difference in energy expenditure per individual walk could summate across shifts to a noteworthy total energy cost across the season. Some king penguin colonies are located two kilometres from the shore, reaching an altitude of more than 100 metres (Guinet et al., 1995, Halsey et al., 2007b). Halsey et al. (2007b) estimated that king penguins consume between 23 and 31l of O_2 to walk two kilometres with a body mass of 10 and 13 kg, respectively. However, Halsey et al. (2007b) did not account for the local topological variations, implicitly assuming a flat topography, nor did they account for the additional energy expenditure of crossing the breeding area, which induces a significant stressed state owing to aggressive conspecifics that protect their territory (Williams, 1995).

To assess the energetic advantage experienced by early breeders, the energy expenditure of both early and late breeders was investigated during the longest fasting period, encountered by the males between the first and second shifts while incubating the egg. Indeed, extension of the fasting period is expected to happen in the next decade due to the predicted

displacement of the polar front. This displacement will increase the distance from and the time taken to reach foraging sites at sea (Peron et al., 2012). Their longest fasting period could become the limiting factor for their reproductive success. The onshore energy expenditures of king penguins have been investigated previously in terms of the energy expenditure of different behaviours while incubating (Viblanc et al., 2012a, Viblanc et al., 2012b, Viblanc et al., 2011a), and, separately, the energy expenditure of walking (Halsey et al., 2007b). However key parameters need to be considered such as the energy expenditure associated with stressed state and the additional energy expenditure of walking on an incline. Therefore, this chapter estimates the energy expenditure during the longest fasting period for early and late breeders, taking the cost of stressed state and walking on an incline into account. Data from previous, studies adding for the cost of stress *per se* measured in chapter three were used to estimate the daily energy expenditure. Data from 22 birds measured when walking on an incline, at different speeds and body masses and data from six walking birds measured while stressed (chapter three) were used to estimate the walking energy expenditure depending on the zone of attachment location.

6.3 Materials and methods

6.3.1 Birds and experimental protocol

6.3.1.1 Walking energy expenditure on an incline

Penguins were captured in the morning and soon afterwards their ability to walk on a treadmill was assessed. When enough penguins suitable for the treadmill had been captured, data collection began. The first experiment generally took place on the same day or the day following capture. The penguins of group D were kept in a pen until the end of experiments (Table 2-1). Before the experiment, each bird was weighed and equipped in the same fashion as the birds used in § 2.2.2.1. The bird was then placed in the respirometer chamber upon a treadmill such that he walked at controlled speeds. The $\dot{V}_{O_2}$ and VeDBA of courting birds were measured as soon as the bird was put in the respirometer chamber. The bird rested for one hour in before the treadmill was turned one, thus requiring the bird to walk (Figure 2.20

for an example of the experiment schedule). Then, an initial walking session of five minutes was completed to acclimate the bird to walking on the treadmill. The experiment involved one set of four walking sessions at speeds of 1, 1.2, 1.4 and 1.6 km/h, with 10 minutes rest between each. The speed order was randomised. One set of walking sessions was conducted on a 13% incline. Experiments and data collection were repeated four times at approximately days 0, 7, 14 and 21, with the respective average body masses referred to as 'heaviest' (13.2 kg), 'heavy'(11.7 kg), 'light'(11.0 kg) and 'lightest' (9.8 kg). Birds were kept in a pen after the experiment and released at the same place in the colony after the fourth experiment.

6.3.2 Data Processing and Statistical analysis

6.3.2.1 Daily energy expenditure during incubating

Time budget

To estimate the daily energy expenditure of an early and a late breeder, the daily time budget of an incubating king penguin by Challet et al. (1994) was used: 67.2% resting, 17.5% for comfort (i.e. preening, shaking, stretching), 7.4% sleeping and 7.9% defence. Extrapolations of the density of the colony, for early and late breeding males whilst incubating, were made from Figure 26 in Viblanc (2011b) (i.e. 150 and 350 birds, early and late, respectively). From Figure 31 (Viblanc, 2011b) reporting the relationship between colony density and resting heart rate, extrapolation of resting heart rate was calculated for an early and a late breeder (i.e. 39 and 47 beats/min, respectively). These values were used to represent heart rate while resting (67.2% of the day) and sleeping (7.4% of the day) and to estimate daily energy expenditure using the calibration relationship reported in Groscolas et al. (2010).

Equation 6-1

$$EE = -387 + 52.5 * HR;$$

where EE is energy expenditure (kJ/day) and HR is heart rate (beats/min). Energy expenditure during comfort activity and defence was calculated from Viblanc et al.(2011a) and VAV's thesis (unpublished work, *in press* or in review) (i.e. 61.2 kJ/h for energy

expenditure of comfort, and 29.8 kJ/h for energy expenditure of aggressive behaviour). Early breeders were assumed to be unstressed, while the late breeders were assumed to be stressed due to the higher density and aggression between birds, and higher measured glucocorticoides (VAV's thesis, Viblanc et al., *in press*). Therefore the energy expenditure of the stress response *per se* (see chapter three) was added to the late breeders' resting metabolic rate: excess heart rate was multiplied by the percentage increase in$\dot{V}_{O_2}$ due to the presence of a stressor (26% increase in $\dot{V}_{O_2}$ while stressed for a similar heart rate ; in birds from group C). As the energy expenditure of defence was estimated from heart rate data (VAV's thesis), daily energy expenditure for both early and late breeders was also multiplied by the percentage of increase in $\dot{V}_{O_2}$ due to the stressor. Total daily energy expenditure was calculated for both early and late breeders and compared.

6.3.2.2 Walking energy expenditure

Energy expenditure of walking

As in Halsey et al. (2007b), $\dot{V}_{O_2}$ as a function of speed was calculated accounting for body mass. This allowed the linear equation of the relationship to be determined as in chapter five: Equation 5-1 with data of birds group D:

$$\dot{V}_{O_2} = a * s + b$$

; where s is speed (m/s). Four different linear equations were investigated using two body masses ('heaviest' mass ~13.2 kg and 'lightest' mass ~9.8 kg) and two inclines (0% flat and 13% incline). Furthermore, the data set obtained while walking under the presence of a stressor was also used (data of bird group C, chapter three) to account for the influence of stressed state on the energy expenditure of walking. Specifically, the energy costs associated with stress *per se* were included, defined as a direct result of the stressor and not including the physiological response to increased body motion as a result of the stressor (i.e. having the same level of motion between unstressed/stressed states).

Topographic and energetic aspects of an onshore roundtrip

The four colonies of (A) Ile aux Cochons (Crozet Archipelago), (B) Ratmanoff (on the Kerguelen Archipelago), (C) Jardin Japonais and (D) La Baie du Marin (both on Possession Island, Crozet Archipelago) were incorporated in the development of the estimate. Using www.geocontext.org/publ/2010/04/profiler/en/, two extreme locations inside the colonies (with the shortest or longest distances/height from the sea) were digitised allowing distance and altitudes values to be imported to R Cran (R Core Team, 2012). King penguins usually avoid walking through the colony and prefer following a path about the periphery using standard routes, where less aggressive interactions therefore occur (Pers. Obs.). However such routes were not discernible from the satellite images, thus journey routes using the periphery of the colony were chosen based on shortest distances. The package "RgoogleMaps" (R Core Team, 2012) was used to create visual representations of the journeys using Google maps. For each colony, the energy expenditure of pedestrian locomotion was then calculated accounting for body mass, topography and stressed state. King penguins were assumed to be at the "heaviest" body mass when walking to the zone of attachment (i.e. having just returned from a foraging trip at sea) and at the "lightest" body mass while walking back to sea. Each journey was split into upward and downward inclines. From each incline, its minimum and maximum were used to calculate the distance and the altitude walked, given by www.geocontex.org. However the degree of incline encountered in the experiments (13 %) often did not represent that encountered in the field. Therefore, if the true incline was greater than 13% the energy expenditure of walking on the incline was considered similar to walking at 13%. When the true incline to be modelled was less than 13% the distance over which the incline existed was conceptually divided into sequences of flat and 13% inclines, which summed to represent that same total altitude achieved over the same horizontal distance walked. For instance, a journey of 10 metres on a 6.5% incline was modelled as a journey of five metres on the flat and five metres on a 13% incline. Energy expenditure when walking on negative inclines (i.e. downhill) was assumed to be the same

as walking on the flat (energy expenditure decreases slowly with angle of downhill slope up to 9% decline, Margaria, 1968). Because data are not available for birds both walking on an incline and subjected to a stressor, when such a situation was encountered in the model it was represented by estimates of energy expenditure obtained for walking on an incline since this represented the highest energy costs recorded in the laboratory. Birds were considered to be in a stressed state when crossing the breeder area. Unfortunately walking energy expenditure while stressed has only been measured at the heaviest body mass (chapter three), thus the energy expenditure at the heaviest body mass while stressed was also used for the return journey through the breeding area. Personal observations (ASTW) indicated that preferred pedestrian speeds are 1.0, 1.2 and 1.4 km/h, on an incline, on the flat, and while stressed, respectively. In the estimate therefore birds were assumed to walk at these speeds in the respective scenarios. Energy expenditure per metre walked taking into account walking speed, body mass, incline and stressed state were used to model the $\dot{V}_{O_2}$ of for each roundtrip. Conversion of oxygen consumption into energy expenditure in Joules was made using the following equation:

Equation 6-2

$$\mathrm{EE} = 20.11 * \dot{V}_{O_2}, \text{ (Schmidt-Nielsen, 1997)}$$

; where EE is the energy expenditure in Joules *per se*cond.

6.3.2.3 Energy expenditure during the longest fasting period

Using the incubating and walking energy expenditures calculated as described in previous sections (§ 6.3.2.1 and § 6.3.2.2), the total energy expenditure of an early and late breeding male between the first and second shift was investigated. Late breeders were considered to have the longest roundtrip in the colony. To assess the importance of walking energy costs to overall onshore energy expenditure, the percentage contribution of walking to daily energy expenditure and for the entire fasting period (20 days in average; Barrat, 1976) was calculated. The different energy expenditures calculated for walking short and long roundtrips were compared.

Table 6-1 Analysis summary. VAV: Vincent A. Viblanc

Aims	Behaviour		Birds type	Analyses	
Energy expenditure of incubating male between the first and second shift, taking the energy expenditure of stress response and incline into account.	***Incubating***	***Resting (67.2% of the time budget) and sleeping (17.5%)***	VAV's data + six incubating birds (group C)	**Early:** 1. Extrapolation of the density and its related resting heart rate found during early breeding period from VAV's figures. 2. Conversion into energy expenditure (Equation 6-1). **Late:** 1. Extrapolation of the density and its related resting heart rate found during late breeding period from VAV's figures. 2. To include the additional energy expenditure of the stress response *per se*, the difference between resting heart rate of an early and late was calculated and multiplied by 126%. 3. Conversion into energy expenditure (Equation 6-1).	
		Comfort (7.4%)	VAV's data	61.2 kJ/hour	
		Defence (7.9%)	VAV's data + six incubating birds (group C)	**Early:** 29.8 kJ/h multiplied by the energy expenditure of the stress response *per se* 126%. **Late:** 29.8 kJ/h multiplied by the energy expenditure of the stress response *per se* 126%.	
	Walking	***Flat***	10 birds in courtship (group D, walking on a flat and on an incline at two different body masses)	***Calculation of the energy expenditure per metre***: Equation 5-1. $EE = a * s + b$	1. Use of www.geocontext.org/publ/2010/04/profiler/en/ to draw journeys of early (i.e. shortest) and later (longest) breeders. Then exportation of distances and altitude data. 2. Calculation of the energy expenditure of each journey using the energy expenditure per metre depending on the terrain and body mass. 3. Conversion of the oxygen consumed into energy expenditure (Equation 6-2).
		Incline			
		Stressed (i.e. walking inside the colony)	six birds in courtship (group B, walking while unstressed and stressed)	***Calculation of the energy expenditure per metre:*** Only one data point.	
	Whole fasting period		All data above	**Early:** Addition of the early breeder daily incubating energy expenditure multiplied for 20days with the walking energy for the shortest roundtrip. Walking energy expenditure was compared in percentage to the daily and fasting incubating expenditure. **Late:** Addition of the late breeder daily incubating energy expenditure multiplied for 20days with the walking energy for the longest roundtrip. Walking energy expenditure was compared in percentage to the daily and fasting incubating expenditure.	

6.4 Results

See Appendices for raw data.

6.4.1.1 Daily energy expenditure during incubating

Daily energy expenditure was estimated to be 1988 kJ and 3311 kJ for an early and a late breeder, respectively (Figure 6.1). These ranges are similar to the daily energy expenditure calculated by Groscolas et al. (2010) based on the body mass loss of king penguins incubating in the colony or while captive in pens (1315 to 5903 kJ). The components of the expenditure were estimated at 1661, 256 and 71 kJ for the resting and sleeping, comfort and defence energy expenditures of an early breeder, and as 2983, 256 and 71 kJ for a late breeder (Figure 6.1). The daily energy expenditure of a late breeder was 66% higher than the early breeder's daily energy expenditure.

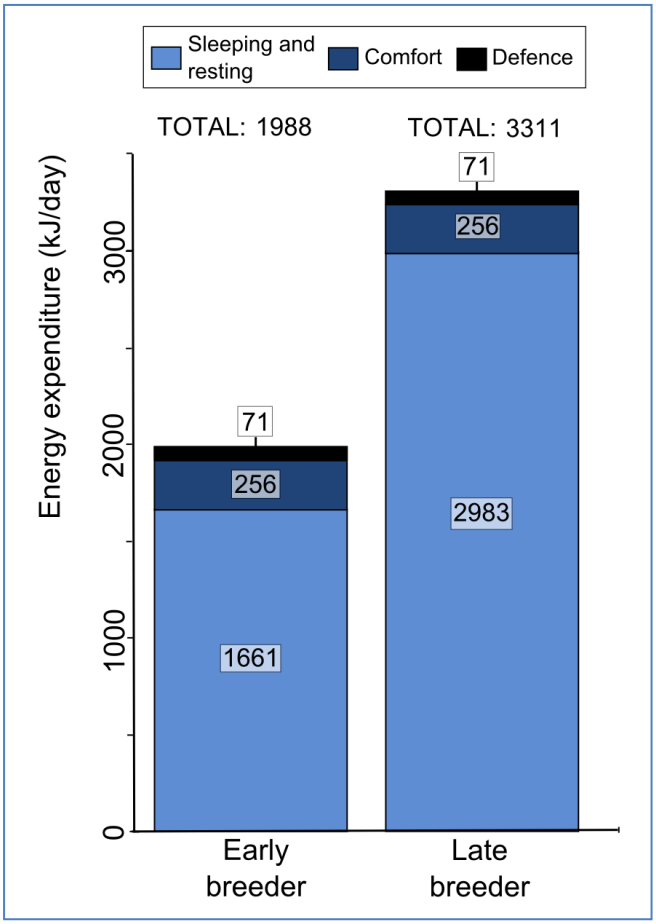


Figure 6.1 Estimation of the daily energy expenditure for an early and late breeder. The light boxes represent the energy expenditure of sleeping and resting (including the energy expenditure of stress response *per se* for the late breeder), the dark boxes are the energy expenditure for comfort behaviour and the black boxes are the energy expenditure for defence behaviours (including the energy expenditure of stress response *per se*).

6.4.2 Walking energy expenditure

6.4.2.1 Energy expenditure of walking

The different linear equations of the relationship between $\dot{V}_{O_2}$ and speed were calculated accounting for body mass, inclines and stressed state and can be seen in Figure 6.2.

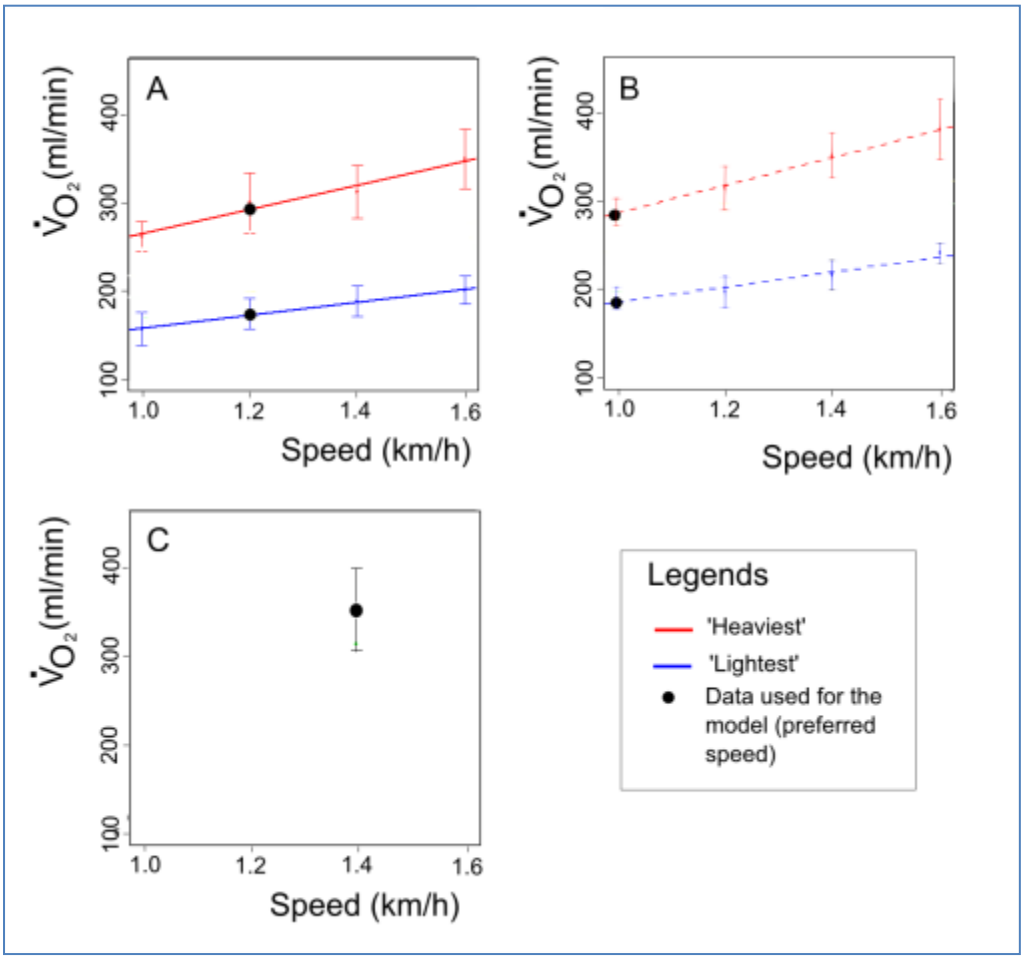


Figure 6.2 $\dot{V}_{O_2}$ – speed relationship as a function of various important parameters. A: 'heaviest' and 'lightest' body mass, walking on the flat. **B:** heaviest' and 'lightest' body mass, walking on an incline. **C:** walking while stressed at "heaviest" body mass. **Legends** The black points highlight the data used in the estimate of energy expenditure for a roundtrip to the colony**.** The different colours represent the two different body masses; see legend box in the graph. Terrain: flat=plain line, incline=dashed line.

The energy expenditure of walking one metre on a flat surface at 1.2 km/h ranged from 174 to 293 kJ, for the lightest (~9.8 kg) to the heaviest (~13.2 kg) body mass (Table 6-2). Walking one metre on an incline cost between 206 to 343 kJ (for the lightest to the heaviest body mass, respectively) and walking one metre while in the colony (thus while stressed)

cost 303 kJ for the heaviest birds (data from chapter three) The flat data corresponded to the range values found by Halsey et al. (2007b) while walking two kilometres on a flat surface.

Table 6-2 Energy expenditure to walk one km (at their preferred speed) in kJ and LO_2 (in brackets)

Body Mass	Flat 1.2km/h	Incline 1km/h	Stressed 1.4km/h
Heaviest (~13.2 kg)	293 (14.59)	343 (17.04)	303 (15.09)
Lightest (~9.8 kg)	174 (8.63)	206 (10.26)	NA

6.4.2.2 Topographic and energetic aspects of an onshore roundtrip

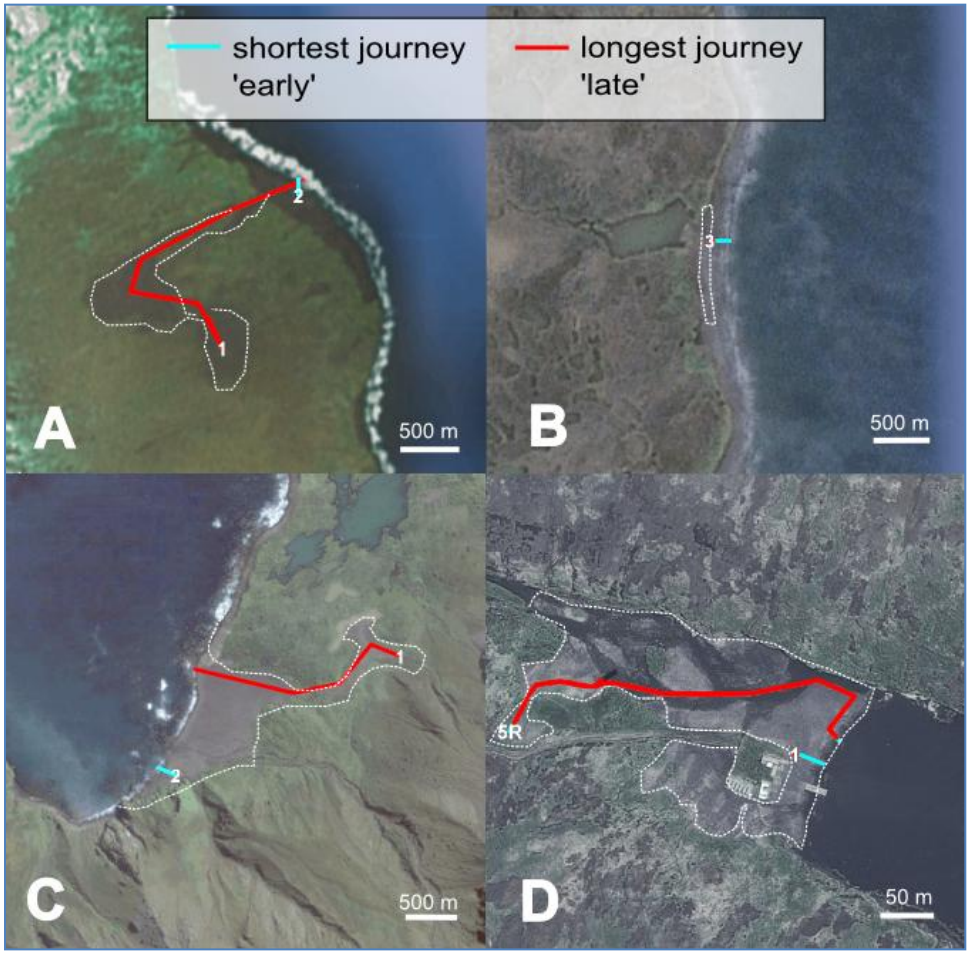


Figure 6.3 Bird's eye view of likely shortest and longest routes into the colony. Colony areas are denoted by a dotted white line. The breeding areas that are crossed are highlighted with a thick white shade around the line. **A.** Ile aux Cochons. **B**. Ratmanoff, Kerguelen. **C.** Jardin Japonais, Possession Island. **D.** La Baie du Marin, Possession Island. The inclines are not highlighted in these images, however, they were taken into account in the estimations of walking energy expenditure. Modified pictures from maps.google.com using R.

The shortest and longest distances overland to the colonies of Ile aux Cochons (A), Ratmanoff (B), Jardin Japonais (C), and Baie du Marin (D) can been seen in Figure 6.3. The longest and shortest distances at Ratmanoff are the same as the colony is parallel to the shore, thus all zones of attachment are roughly the same distance to the sea (Figure 6.3). Furthermore, as the colony is on the coastline, there is negligible incline. A topographic profile of the journeys can be seen in Figure 6.4.

The distance walked ranged from 59 to 311 m and between 848 to 5209 m, for shortest and longest roundtrips respectively across the four modelled colonies (Table 6-3). The energy expenditure for the shortest roundtrips ranged between 17 to 94 kJ, while the range of energy expenditure for the longest roundtrip was between 225 and 1321 kJ (Table 6-3 and Figure 6.1).

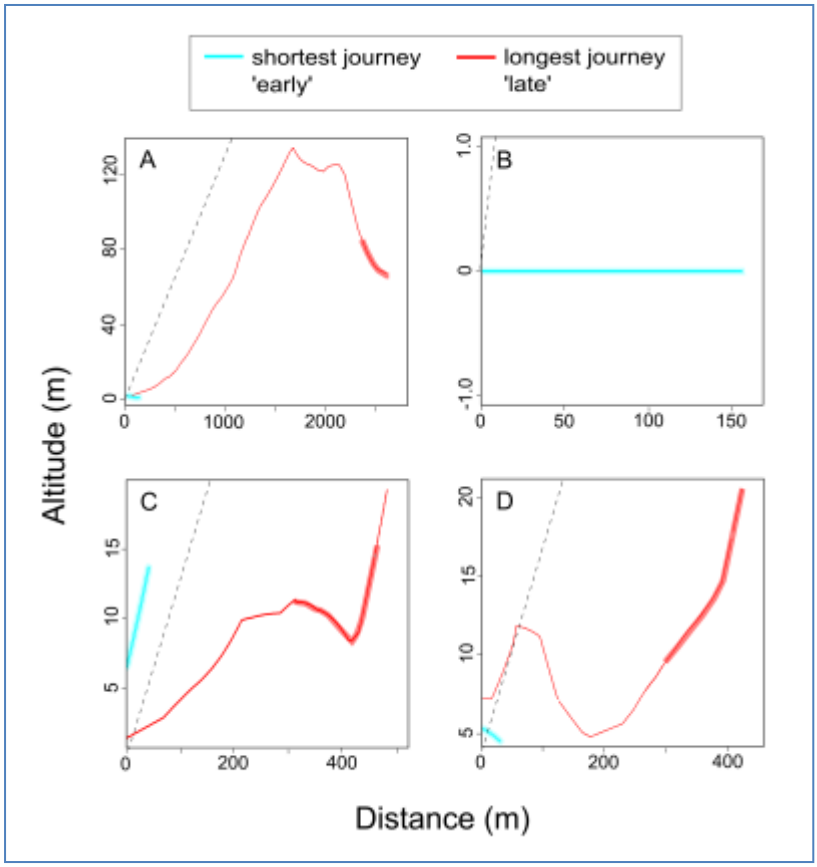


Figure 6.4 Topographic profile of likely shortest and longest journeys into various colonies. **A.** Ile aux Cochons. **B**. Ratmanoff, Kerguelen. **C.** Jardin Japonais, Possession Island. **D.** La Baie du Marin, Possession Island. The breeding areas are highlighted by thickened lines. The dashed lines represent a constant 13% incline.

Table 6-3 Energy expenditure of likely shortest and longest roundtrips to a colony by king penguins. Early breeders were assumed to undertake the short roundtrip and the late breeders were assumed to undertake the long roundtrip. 'Short' and 'Long' refer to roundtrips, 'early' and 'late' refer to the breeders.

Colony		A. Ile aux Cochons		B. Ratmanoff, Kerguelen		C. Jardin Japonais, Possession Island		D. La Baie du Marin, Possession Island	
Journey (return)		Short. 'early'	Long. 'late'	Short. 'early'	Long. 'late'	Short. 'early'	Long. 'late'	Short. 'early'	Long. 'late'
Energy expenditure (kJ) (Distance in m)	Flat	0 (0)	697 (3114)	0 (0)	0 (0)	0 (0)	109 (497)	0 (0)	83 (380)
	Incline	2 (10)	469 (1585)	0 (0)	0 (0)	14 (41)	53 (164)	1 (6)	64 (210)
	Stressed	77 (255)	155 (511)	94 (311)	94 (311)	12 (41)	92 (305)	16 (53)	78 (258)
	TOTAL	**79 (265)**	**1321 (5209)**	**94 (311)**	**94 (311)**	**26 (81)**	**254 (966)**	**17 (59)**	**225 (848)**

6.4.2.3 Energy expenditure during the longest fasting period

The estimated energy expenditure while incubating for the 20-day fasting period between the first and second shift cost between 39789 and 39865 kJ and between 6639 and 67535 kJ, for early and late breeders, respectively (Table 6-4). Roundtrips represented 1 to 5%, and 7 to 41% of the daily incubating energy expenditure for the shortest and longest roundtrip, respectively. Roundtrips represented 0.04 to 0.2%, and 0.1 to 2% of the total energy expenditure over the 20-days ashore, incorporating the shortest and longest roundtrips to and from the colony, respectively (Table 6-4).

Table 6-4 Energy expenditure estimation for late and early breeders between the 1st and 2nd shift, at different colonies. EE is for energy expenditure. Walk is for Walking and Inc for incubating. Late breeders are assumed to undertake the longer routes to and from the colony.

Colony		A. Ile aux Cochons		B. Ratmanoff, Kerguelen		C. Jardin Japonais, Possession Island		D. La Baie du Marin, Possession Island	
Breeder		Early	Late	Early	Late	Early	Late	Early	Late
Energy expenditure (EE in kJ)	Incubating (daily)	1988	3311	1988	3311	1988	3311	1988	3311
	Walking (return)	79	1321	94	94	26	254	17	225
	Total for 20 days	**39850**	**67535**	**39865**	**66308**	**39798**	**66468**	**39789**	**66439**
Percentage $\left(\frac{EEwalk}{EEinc} * 100\right)$	Daily	4	40	5	3	1	8	1	7
	Fasting period (20 days)	0.2	2	0.2	0.1	0.07	0.4	0.04	0.3

6.5 Discussion

The estimated daily energy expenditure of late breeders, while incubating between the first and the second shift, is 66% higher than the daily energy expenditure of early breeders (1984 and 3311, for early and late breeders respectively), indicating a considerable energetic disadvantage for late breeders. This is explained mostly by an increase in aggressiveness between neighbours linked to the increase in colony density (resting heart rate 39 and 47 beats/min for early and late breeders, respectively, representing 1661 and 2081 kJ, without accounting for the additional cost due to stress *per se*). Consequently, the energetic equivalent of an average 20 days fasting period for an early breeder is just 12 days for a late breeder, resulting in a temporal disadvantage of eight days for the late breeders.

The maximal difference in cost of walking between early and late breeders within the same colony (i.e. assuming that the early breeders take short routes and late breeders take long routes, and that late breeders are obliged to take zones of attachment at higher altitudes and experience greater stressed state while walking to them) represents an increase of 36% of the daily incubating energy expenditures (4 to 40%, in Ile aux cochons), and 1.8% of the total energy expended during a 20-day fast (from 0.2 to 2%). Thus, the longer roundtrip is estimated to represent a reduction in maximal fasting duration of less than half a day. Thus it might be reasonable to conclude that the difference in reproductive success between early and late breeders is not generally due to additional costs of a longer or for other reasons energetically more costly roundtrip for the latter, even when accumulated over the breeding season.

In addition, comparison of the walking energy expenditure between the different colonies showed a difference of 36% for a late breeder daily energy budget (3 to 40% for a late king penguin at Ratmanoff and Iles aux cochons, respectively) which only represents a 1.9% different in the 20-days energy budget (from 0.1 to 2%). These results suggested that the pedestrian locomotion energy expenditure due to the choice of colony (at least between the

specific four measured colonies) is not an important parameter affecting their incubating energy budget, representing only a temporal advantage of a third (36%) of a day of a 20-day fasting period.

Furthermore, the present results showed that the energy expenditure of walking only represented a maximum of 40% of an incubating late breeder's daily energy expenditure, which represented 2% of an incubating late breeder 20-day energy expenditure. This suggests that, generally, the energy expenditure is not an important parameter affecting the fasting energy budget. Even when taking the cost of the stress response *per se* and incline into account, the present results support the previous finding that the energy expenditure of walking for a roundtrip is unlikely to be subject to selection pressures (Halsey et al., 2007b, Angelier et al., 2006).

While the longest incubating fasting period by king penguins is known to last 20 days on average (Barrat, 1976), this represents both early and late breeders together; data are not presently available for these groups of birds separately. Clearly then, future work should record observed durations of incubating between two shifts for early and late breeders separately as well as the fasting duration until egg abandon. From the predictions of the present model, late breeders are able to remain fasting ashore for eight days less. This is a dramatic difference and thus could well be a contributory factor to the lower reproductive success rates of late breeders in the event of unfavourable year. Furthermore, this difference may become even greater due to the potential increase in the distance out to sea that king penguins must travel to reach foraging areas, predicted to be up to 25 to 40 km per decade (Peron et al., 2012) due to displacement toward the south pole of the polar front. Indeed, this displacement of foraging patches in response to global warming may result in an additional day every decade associated with each foraging trip (Charles-André Bost, unpublished data). This may result in the available fasting duration becoming a limiting factor for king penguin

reproductive success. Thus improving the knowledge of incubating energy budget of early and late king penguin may enable a better forecast of king penguin population viability in the face ofglobal warming.

The model developed in this study of course included a number of simplifying assumption. For example, the cost of stress *per se* may be different according to the stressor (Moberg and Mench, 2000). Here, the energetic values used to represent the costs of stressed state were collected from experiments employing an anthropogenic stressor, which possibly may not be particularly generalisable. In addition, acclimation time may last longer than the defence event itself, meaning that the extra cost due to the stress response *per se* may continue beyond the defence duration, which would increase the daily energy expenditure. To account for incline walking, data were used to parameterise the model based on a single incline angle only, and no downward incline. The resting heart rate included in the model, taken from Viblanc (2011b)'s data, represents the lowest heart rate reported and is thus assumed to represent the minimal energy expenditure of an incubating king penguin. These birds may sometimes exhibit resting and sleeping behaviour requiring a higher metabolic rate than this minimum value for a variety of reasons including slight changes in physiological state or activity level. For instance, Viblanc showed in his thesis (2011) that heart rate changes during the breeding cycle, having, for example, a peak a few days before the laying day for early breeders. Furthermore, the model focuses on energy expenditure. However the energy reserves may be different between groups of birds, such as there might be differences in weight and body condition (proportions of fat to protein) between early and late breeders.

Despite these limitations, this study demonstrated the application of using energy expenditure to better understand the ecology of a species. The results of this present chapter offer evidence that there is a considerable energetic advantage to being an early breeder, at least during the first, and longest, incubating fasting period by the male.

7. General Discussion

7.1 Summarising the results and further work

Measuring the energy expenditure of a species is key to better understanding its life history (Hall et al., 2001), trophic flow (Lowe, 2002), biogeography (McNab, 2002) and behavioural strategies (Hinch and Rand, 1998), as mentioned by Gleiss et al. (2010). By investigating the energy expenditure of king penguins, this thesis generates new insights not only into their physiological stress response and the biomechanics of pedestrian locomotion, but also into proxy-based methods of measuring energy expenditure.

7.1.1 The physiological stress response of king penguins

Techniques for measuring energy expenditure via respirometric calibrations of measurable proxies, in particular heart rate and accelerometry, are increasingly being used yet there is still considerable scope for enhancing their predictive accuracy through rigorous experimentation and validation. For example, while there are a plethora of calibration studies refining the relationship between $\dot{V}_{O_2}$ and heart rate in king penguins, to date no published work has explicitly investigated the effects of a laboratory-induced stressed state. In order to improve the accuracy of energy expenditure estimations of king penguin, this thesis looked at the effect of the presence of a stressor on their physiology. Through knowing the stress response of a king penguin, it is possible to identify the potential bias of data collected from a bird not fully acclimated to its experimental surroundings or to the protocol during calibration experiments. However, stress responses are difficult to generalise (Romero, 2004). Therefore chapter three sought to determine the cardio-respiratory and behavioural responses of king penguins in the specific context of the presence of an anthropogenic stressor.

Cardio-respiratory stress responses in king penguins taking the movement into account

The nature of responses to a stressor depends on several parameters, such as the species in question (Hill et al., 2008), individuals within a species (Romero, 2004) (which can differ for a number of reasons including variation in life history) and the type of stressor involved (Moberg and Mench, 2000). Nonetheless, while determining cardio-respiratory and

behavioural stress response, movements are a clear confound. In these studies, it was found that the stress responses were partly due to an increase in activity level (i.e. behavioural stress response: 'fight or flight' response), thus this issue was investigated in depth here. The results showed the importance of factoring in the movement (i.e. activity and motion) of the subject animal during the presence of the stressor. Indeed the cardio-respiratory and behavioural stress responses are different depending on whether the animal is active while stressed or not, and on whether the measured response is based on the overall or *per se* stress response. The overall cardio-respiratory and behavioural stress response of an active king penguin results in a significant increase in mean $\dot{V}_{O_2}$ only, while the stress response at low activity is an increase in mean $\dot{V}_{O_2}$, heart rate and VeDBA. When the stress response *per se* is measured (i.e. the physiological and behavioural stress responses which are a direct result of the stressor, without including the physiological response due to increased body motion), $\dot{V}_{O_2}$ is the only parameter which increases significantly. In addition, these results also demonstrate for the first time the short term cost of the stress response. This chapter demonstrates that noteworthy error can be introduced if a subject animal is not fully acclimated during a calibration experiment.

Stress-induced biases

Developing on from chapter three, chapter four proposed a protocol to reduce the stress-related bias induced by exposure of a king penguin to treadmill-calibration experiments. Leaving the bird acclimating for 90 minutes in the respirometer chamber and walking them for a session prior to data collection on a treadmill was shown to remove much of the stress-related error in measurements. Future work should include simultaneous hormone analysis to better describe the levels of acclimation achieved (Romero, 2004); however, this analysis must not interfere with the natural behaviour of the animals and, of course, not increase the stress response. By measuring the cardio-respiratory system alone, nonetheless the results of

chapter four showed that with an appropriate acclimation, it is possible to effectively attenuate the stress related confound.

7.1.2 The biomechanics of pedestrian locomotion in king penguins

Understanding GCOT

To date, the parameters influencing GCOT and how they interact are uncertain. While principal parameters, such as stride frequency, have been defined, others, such as body mass have been shown to have contradictory influences on GCOT, and it is not fully understood why this is the case. Although partitioning GCOT has shown good results in improving this understanding (Steudel, 1990, Marsh et al., 2004, Halsey, 2013), the results presented in chapter five have been inconclusive. The small sample size in the biomechanical data may have influenced these findings. Further research is also needed to test the hypothesis of sagital displacement of the centre of mass in heavy king penguins. However, chapter five is a step towards better understanding GCOT through partitioning it into NCOT and PCOT.

7.1.3 From the laboratory to the field: ecological energetics

Due to the predicted future displacement of the polar front, the distance and time taken by king penguins to reach their foraging sites at sea will be extended (Peron et al., 2012). Consequently, the longest fasting period of the king penguin – the first incubation phase by the male - is likely to become the limiting factor for their reproductive success. Furthermore, investigations of the different energy budget management between early and late breeders could enable a better understanding of their differing reproductive success. Therefore measurements of energy expenditure obtained through this project were used to estimate the energy use between early and late breeders while incubating between shift one and two (chapter six). Despite some simplifications made in the estimate as, for instance, applying the energy cost recorded for an anthropogenic stressor to represent stresses in the field, noteworthy differences in energy expenditure between early and late breeders were found. The higher stressed state encountered by late breeders due to the higher density of the colony at the start of the breeding season results in them having an estimated 63% higher incubating

energy expenditure per day than early breeders. This suggests that their reproductive success is more likely to decrease if the daily energy budget is restricted (for instance extension of the fasting period, or increase of energy expenditure due to an anthropogenic presence) than is the case for early breeders. Late breeders are also likely to have to walk further and / or higher to reach their zone of attachment. It was calculated that the associated energy expenditures of locomotion represented only up to 2% of the total energy expended over a 20-day incubating period, suggesting that the additional cost of walking for a late breeder is not so important for their reproductive success, at least in term of energy budget. Similarly, the small difference in walking energy expenditure between colonies representing locations of very differing shape, size, distance from the coast and altitude (maximum energy costs difference of 1.8%) suggests that the choice of location of the colony for incubating is also not an important factor concerning king penguin reproductive success.

7.2 Relevance and application of the findings

7.2.1 Understanding the cardio-respiratory stress response *per se*

For the king penguin model at least, if heart rate or $\dot{V}_{O_2}$ are used as measures of stress, it is imperative that movements are accounted for. It would appear that the increase in measured heart rate might be due to the increased oxygen demands of active muscles (change in motion) rather than as a direct result of the physiological stress response *per se*. If the findings for king penguins are generalizable to other species then without measures of activity there is likely to be spurious interpretations of wellbeing if body movement is not considered (von Borell et al., 2007).

7.2.2 Energetic costs of being stressed in the short-term

Furthermore, while the disturbance of penguins by anthropogenic factors has already been demonstrated and studied (Culik and Wilson, 1991, Nimon et al., 1995, Culik and Wilson, 1995, Viblanc et al., 2012a), the results in chapter three demonstrated that stressors have an energetic impact, as mean $\dot{V}_{O_2}$ significantly increased during the stressed state. Possibly then the cost of short-term stress responses should be taken into account when considering

conservation issues. The decreasing size of the population of the colony at La Baie du Marin, Possession Island, where the field work for this study was conducted, could partly be due to this anthropogenic presence. This is the only colony on the island to have a human presence throughout the year and also the only colony to be decreasing in numbers (Viblanc et al., 2012a, Delord et al., 2004). Factoring in the additional energy expenditure and its duration due to human presence (punctual or constant) could enable quantification of its impact on overall energy expenditure. The influence of this result could be evaluated in relation to other factors which may also be influencing the current population decrease, such as habitat loss. Such models could improve the management of penguin colonies which experience a human presence, or more generally, any place where humans interact with the fauna.

7.2.3 Reassessing previous results taking stress state levels into account

The results also suggest that stress-induced error may exist in previous calibration experiments which did not allow sufficient acclimation by the subject birds prior to measurements being taken. For instance, interpreting research on the energy expenditure of king penguins at sea (Butler, 2006, Halsey and Butler, 2006) in light of the above could encourage some reinterpretation of the results. Estimated energy expenditure of king penguins at sea enabled calculation of the aerobic dive limit, which is the ratio of usable oxygen stores to $\dot{V}_{O_2}$ during diving (Butler, 2006), representing the theoretical limit to aerobic dive duration. In several species, including king penguins, animals have been shown to exceed this limit. For example, 20% of king penguin dives exceed their calculated aerobic dive limit (Butler, 2006). An incorrect estimation of the usable oxygen stores, or an incorrect estimation of $\dot{V}_{O_2}$ consumed during the dive, have been suggested as explanations. Butler (2006) suggested that the usable oxygen store was underestimated due to underestimation of the ATP available from phosphocreatine stores. In the case of king penguins, all measurements of $\dot{V}_{O_2}$ have been made in water channels (Culik et al., 1996, Halsey et al.,

2007a) without mention of an acclimation time. As shown by the results of chapter three, a stressed king penguin has an increased $\dot{V}_{O_2}$. Thus if acclimation to remove this stress-induced confound is not included in the protocol, the calculated aerobic dive limit is estimated from the $\dot{V}_{O_2}$ of a stressed bird and an underestimated calculation of aerobic dive limit would probably result.

7.2.4 Experimental biology: the need to account for stress state

In a more general context, the results of chapter three and four showed that it is important to acclimate the animal to the laboratory and experimental protocol, to obtain more accurate results. This observation is pertinent to measures over and above energy expenditure, especially as stress responses are known to affect almost all physiological systems (Romero, 2004).

7.2.5 The use of accelerometry in biomechanics analyses

Through the research conducted in chapter five, advances were made concerning the methodology for collecting walking data. The video- and accelerometer- based results regarding stride frequency suggest that accelerometers can be used to describe effectively walking gait. The well-known, well-validated and well-used technique of 3D reconstruction of the gait for its analysis (Abourachid et al., 2011, Provini et al., 2012, Provini et al., 2013) was used to quantify the temporal-spatial characteristics of the penguin gait. Thereafter, further analysis of the accelerometry data characterised the acceleration pattern in terms of amplitude and between the axes. This enabled comparison of the animal's walking, and the data could improve the quantification of the change in gait between different body conditions, for instance, high or low body mass. This new method of analysing gait could overcome the issues encountered during field work, which lead to small sample sizes. Furthermore, using the accelerometer output for a first interpretation of results could provide a direction for future work using video recordings in the field. This would potentially help to reduce digitising of video footage for analysis, which is precise but time-consuming.

7.2.6 Understanding GCOT: using incline and a larger range of speeds

Another application of the validated video and accelerometer analysis could be to investigate how gait changes across different speeds and with whether the bird is walking on a flat surface or on an incline. Analysing the biomechanics and energetic changes along with these parameters could lead to additional information which could aid in better understanding of GCOT.

7.2.7 Evaluation of the health of a penguin colony using the biomechanics of pedestrian locomotion

Knowledge about the biomechanics of penguin pedestrian locomotion could underpin applied research. Conservation studies often require non-invasive data collection with a minimum level of human impact. Video recordings of bird colonies are used to monitor behaviour and colony 'health'. Automated recording of arrivals to and departures from a colony is currently being conducted by Tom Hart (from Oxford University). From these data population trends within and over years can be calculated. Ideally, if the penguin gait changes with differing body mass, this could potentially be recognised on videos. Then, from short video sequences taken throughout the year, the nutritional state of a penguin could be determined, without the bottleneck effect that a weigh bridge can bring (Le Maho et al., 1993). This would enable automation of the measurements of key population parameters with less human intervention.

7.2.8 Reducing human impact in a wild colony: using energy expenditure as a unit of comparison

Better understanding of the different energy expenditures of different anthropogenic stressors, and the consequences of those energy expenditures for different birds and at different times of the year/breeding cycle, could facilitate improved cohabitation. For instance, further research on the acclimation time required for both constant and periodic human presence could directly benefit the population of La Baie du Marin, by defining the best frequency and duration of access by people to the colony. The use of accurate measures of onshore energy expenditure, i.e. accounting for oft-neglected factors such as stress state

and its duration, assessed simultaneously with topographic information, could help uncover how energy expenditure varies with location of the 'zone of attachment' within the colony. The results of such on energy landscape, with the collaboration of the French Polar Institute (IPEV), could potentially lead to a more appropriate use of the colony for anthropogenic purposes, taking the wellbeing of the birds into consideration.

7.3 Conclusion

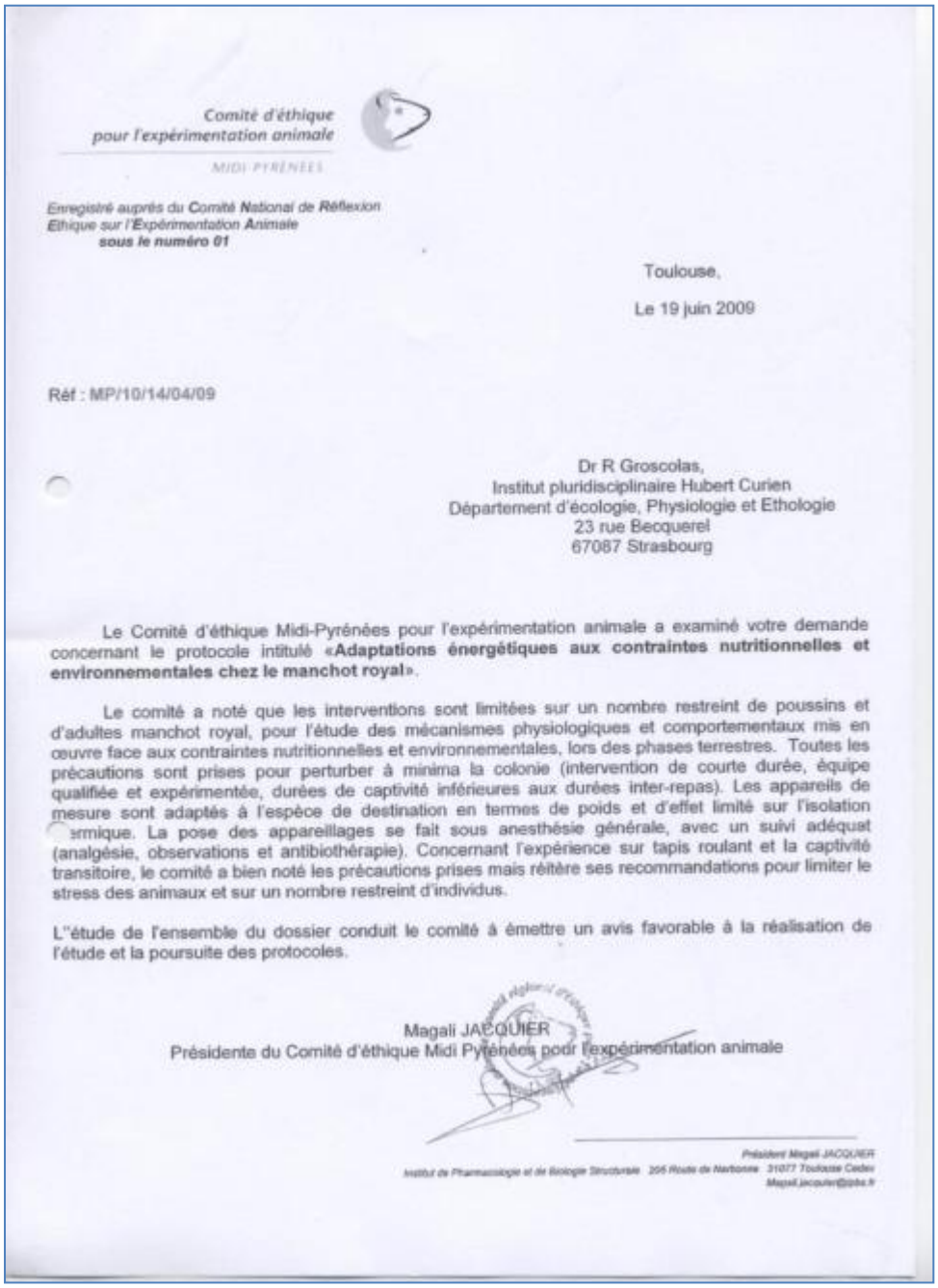
Knowing how animals expend energy is crucial to improving our knowledge of a species. The importance of research on energetics has already been demonstrated (Hall et al., 2001, Lowe, 2002, McNab, 2002, Hinch and Rand, 1998). In addition, this collection of studies has revealed the necessity to take the stress response into account, especially during calibration experiments. Using accelerometer data has demonstrated that taking movement into account is important when defining the stress response of an animal. Despite inconclusive results regarding the mass-independent NCOT of king penguins, the present study is a step forward in defining the parameters influencing GCOT, by suggesting and using a new methodological approach for further research (i.e. partitioning GCOT into NCOT and PCOT and the use of accelerometers in characterising the gait). Finally, including the additional energy expenditure incurred due to stressed state and to walking on an incline has enabled a better understanding of the energy budgets of early and late breeders, which can feed into conservation projects for king penguins.

In summary, the results of this thesis demonstrated the utility of energy expenditure measurements, and the numerous applications that the simultaneous use of respirometry, heart rate and accelerometry data can have to answer interdisciplinary questions.

8.Appendices

8.1 Ethics form

Figure 8.1 Ethical authorisation for the first field work 2009



Comité d'éthique
pour l'expérimentation animale
MIDI-PYRÉNÉES

Enregistré auprès du Comité National de Réflexion
Ethique sur l'Expérimentation Animale
sous le numéro 01

Toulouse,

Le 19 juin 2009

Réf : MP/10/14/04/09

Dr R Groscolas,
Institut pluridisciplinaire Hubert Curien
Département d'écologie, Physiologie et Ethologie
23 rue Becquerel
67087 Strasbourg

Le Comité d'éthique Midi-Pyrénées pour l'expérimentation animale a examiné votre demande concernant le protocole intitulé «**Adaptations énergétiques aux contraintes nutritionnelles et environnementales chez le manchot royal**».

Le comité a noté que les interventions sont limitées sur un nombre restreint de poussins et d'adultes manchot royal, pour l'étude des mécanismes physiologiques et comportementaux mis en œuvre face aux contraintes nutritionnelles et environnementales, lors des phases terrestres. Toutes les précautions sont prises pour perturber à minima la colonie (intervention de courte durée, équipe qualifiée et expérimentée, durées de captivité inférieures aux durées inter-repas). Les appareils de mesure sont adaptés à l'espèce de destination en termes de poids et d'effet limité sur l'isolation thermique. La pose des appareillages se fait sous anesthésie générale, avec un suivi adéquat (analgésie, observations et antibiothérapie). Concernant l'expérience sur tapis roulant et la captivité transitoire, le comité a bien noté les précautions prises mais réitère ses recommandations pour limiter le stress des animaux et sur un nombre restreint d'individus.

L''étude de l'ensemble du dossier conduit le comité à émettre un avis favorable à la réalisation de l'étude et la poursuite des protocoles.

Magali JACQUIER
Présidente du Comité d'éthique Midi Pyrénées pour l'expérimentation animale

Président Magali JACQUIER
Institut de Pharmacologie et de Biologie Structurale 205 Route de Narbonne 31077 Toulouse Cedex
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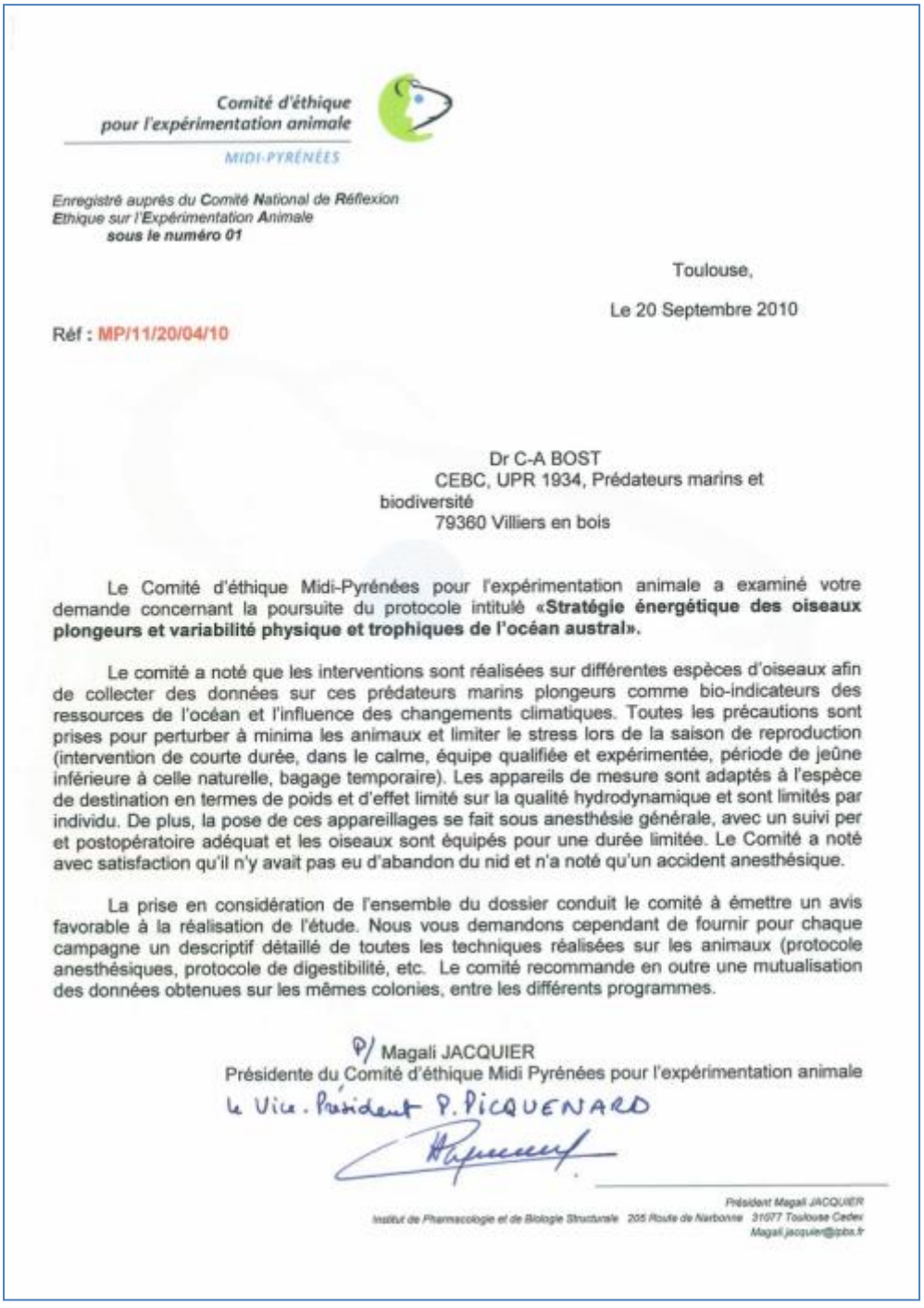
Figure 8.2 Ethical authorisation for the second field work 2010



Comité d'éthique
pour l'expérimentation animale
MIDI-PYRÉNÉES

Enregistré auprès du Comité National de Réflexion
Ethique sur l'Expérimentation Animale
sous le numéro 01

Toulouse,

Le 20 Septembre 2010

Réf : MP/11/20/04/10

Dr C-A BOST
CEBC, UPR 1934, Prédateurs marins et
biodiversité
79360 Villiers en bois

Le Comité d'éthique Midi-Pyrénées pour l'expérimentation animale a examiné votre demande concernant la poursuite du protocole intitulé **«Stratégie énergétique des oiseaux plongeurs et variabilité physique et trophiques de l'océan austral».**

Le comité a noté que les interventions sont réalisées sur différentes espèces d'oiseaux afin de collecter des données sur ces prédateurs marins plongeurs comme bio-indicateurs des ressources de l'océan et l'influence des changements climatiques. Toutes les précautions sont prises pour perturber à minima les animaux et limiter le stress lors de la saison de reproduction (intervention de courte durée, dans le calme, équipe qualifiée et expérimentée, période de jeûne inférieure à celle naturelle, bagage temporaire). Les appareils de mesure sont adaptés à l'espèce de destination en termes de poids et d'effet limité sur la qualité hydrodynamique et sont limités par individu. De plus, la pose de ces appareillages se fait sous anesthésie générale, avec un suivi per et postopératoire adéquat et les oiseaux sont équipés pour une durée limitée. Le Comité a noté avec satisfaction qu'il n'y avait pas eu d'abandon du nid et n'a noté qu'un accident anesthésique.

La prise en considération de l'ensemble du dossier conduit le comité à émettre un avis favorable à la réalisation de l'étude. Nous vous demandons cependant de fournir pour chaque campagne un descriptif détaillé de toutes les techniques réalisées sur les animaux (protocole anesthésiques, protocole de digestibilité, etc. Le comité recommande en outre une mutualisation des données obtenues sur les mêmes colonies, entre les différents programmes.

P/ Magali JACQUIER
Présidente du Comité d'éthique Midi Pyrénées pour l'expérimentation animale
le Vice. Président P. PICQUENARD

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8.2 Data for each chapter

8.2.1.1 Rough Data:

In two summer field trips, the following data were collected:

More than 400 hours of heart rate data at a frequency of 1Hz (in just under 200 files). More than 400 hours of acceleration data at a frequency of 32.5Hz (in just under 200 files). Approximately 200 hours of data to calculate oxygen consumption at a frequency of 1Hz (in approximately 90 files). Just under 6 hours of video taken at 50Hz to analyse king penguin gait (in more than 700 files).

Table 8-1 General abbreviations

Abbreviations	Definitions
Ind	Individual identity
$\dot{V}_{O_2}$[ml/min]	Rate of oxygen consumption in millilitre per minute
HR [beats/min]	Heart rate frequency in beats per minute
VeDBA [g]	Vectoriel Dynamic Body Acceleration in metre per second square

8.2.2 Reassessment of the cardio-respiratory stress response: accounting for movement

8.2.2.1 High activity

Table 8-2 Data of $\dot{V}_{O_2}$ heart rate (HR) and VeDBA of king penguins while walking on the treadmill under different stressing conditions. Stressi:1=unstressed, 2= stressed.

Ind	Stressi	$\dot{V}_{O_2}$[ml/min]	HR [beats/min]	VeDBA [g]
31	1	286.98	143.00	2.99
31	2	310.17	155.50	2.90
32	1	282.94	115.50	2.99
32	2	293.74	114.00	2.96
33	1	253.07	137.50	2.49
33	2	257.28	132.50	2.33
35	1	289.01	180.50	2.66
35	2	371.16	163.50	2.97
36	1	304.24	196.50	2.54
36	2	363.09	190.00	3.04
37	1	283.49	145.50	2.97
37	2	342.68	155.00	3.39

8.2.2.2 Low activity

Table 8-3 Data of $\dot{V}_{O_2}$ heart rate (HR) and VeDBA of incubating king penguins under different stressing conditions. VeDBAg: 1=unstressed same VeDBA as stressed (i.e 2); 2 = stressed; 3 = unstressed different.

Ind	VeDBAg	$\dot{V}_{O_2}$[ml/min]	HR [beats/min]	VeDBA [g]
24	1	64.85	85.89	0.12
24	2	93.34	91	0.12
24	3	55.22	63.12	0.02
25	1	75.33	88.01	0.07

25	2	68.87	104	0.07
25	3	48.34	87.69	0.04
26	1	81.1	128.16	0.2
26	2	123.78	129	0.2
26	3	43.91	67.34	0.04
27	1	89.83	128.5	0.2
27	2	119.28	155	0.2
27	3	49.92	100.56	0.04
28	1	67.71	94.74	0.15
28	2	75.54	82	0.15
28	3	44.46	38.94	0.07
30	1	74.16	73.67	0.17
30	2	91.34	87	0.17
30	3	49.74	33.92	0.05

8.2.3 Avoiding laboratory stress-induced confounds during respirometry: let the king penguin acclimate

8.2.3.1 Introduction: Illustration of the effect of stress on the calibration relationship with the data collected in this thesis.

Two graphs of $\dot{V}_{O_2}$ in function of heart rate from the data used in this thesis are shown. Data were separated into two figures, at high (Figure 8.3) and low (Figure 8.4) activity to improve the fit of the calibrations. Data used in chapter three (Table 8-2 & Table 8-3) and five (Table 8-8) were shown (total n= 153) in the graphs, too. Two calibration relationships were calculated from the data: One including all the data from the thesis apart from the one measured while in the presence of the human stressor (black triangles), and the second one was calculated using only the "unstressed" data from chapter three (light and dark grey triangles). Calibration relationships of king penguins from previous studies (i.e. Groscolas et al. 2010, dotted line; Fahlman et al. 2004 dashed line) are also shown. These graphs have been made for illustrative purposes only. As discussed in chapter four, the calibration relationship of Groscolas et al. (2010) used a time scale measurement of one day, while Fahlman et al. (2004) used birds in courtship. Those different parameters enable comparisons between previous calibration relationships relative to the data measured while stressed, obtained in this thesis (black triangles). Comparison between the calibration relationship obtained with the data of this thesis (dark plain line) and stressed data from chapter three (black triangles) need to be interpreted with caution. Indeed the calibration was

made using a data set (grey dots) with a protocol including only 60min of acclimation, which has been shown in chapter four to be insufficient to remove the bias due to stressor. Thus the calibration relationship itself is potentially biased by the stress effect (dark plain line). Furthermore, regarding the birds at low activity (Figure 8.4), data in this Figure comes from birds at two different reproductive states (incubating for the triangles or in courtship for the grey dots), which may bias the comparison. Consequently, a second calibration relationship has been made with the data measured while unstressed (total n= 18), to represent the bias of the effect of the stress response (see black triangles in comparison to the light plain line). Comparison from this calibration relationship made with "unstressed" data with the "stressed" data shows the displacement of the "stressed" data above the calibration relationships, illustrating the additional cost of stress response.

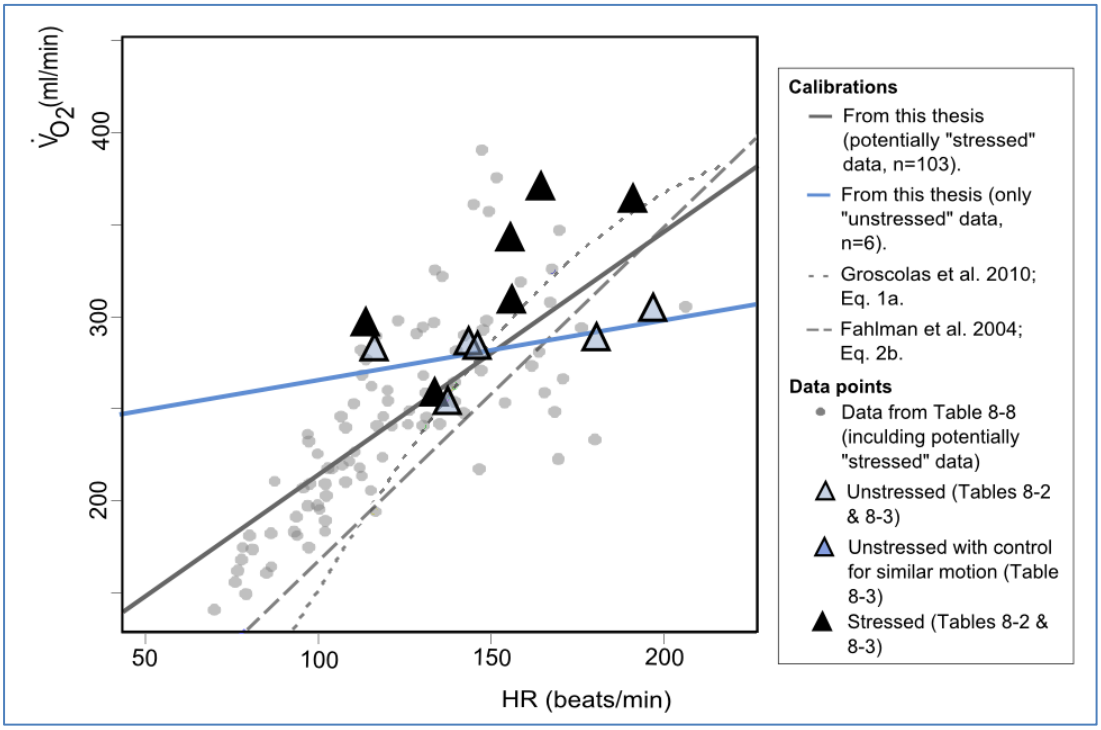

Figure 8.3 Effect of the stress response on the calibration relationships for birds at high activity. Data used in chapter three at high activity (while unstressed –light triangles-, and while stressed –black triangles-) were placed with the calibration relationship calculated with all data used in this thesis while highly active (grey dots and light triangles), apart from while stressed (black triangles). Previous calibration relationships from Groscolas et al. (2010); Eq. 1a (dotted line) and Fahlman et al. (2004); Eq. 2b were also drawn. This graph has been made for illustrative purpose only (see text).

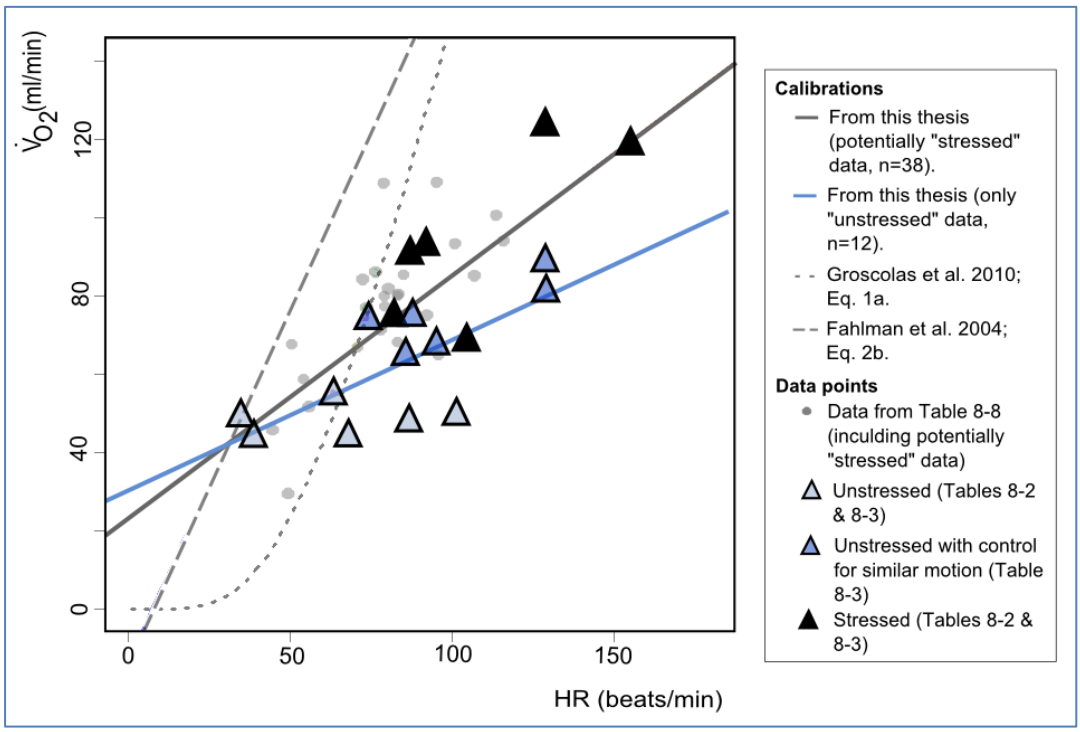

Figure 8.4 Effect of the stress response on the calibration relationships for birds at low activity. Data used in chapter three (while unstressed –light triangles-, while unstressed with control for similar motion-dark triangles- and while stressed –black triangles-) were placed with the calibration relationship calculated with all data used in this thesis at low activity (grey dots, light and dark triangles), apart from while stressed (black triangles). Previous calibration relationships from Groscolas et al. (2010); Eq. 1a (dotted line) and Fahlman et al. (2004); Eq. 2b were also drawn. This graph has been made for illustrative purpose only (see text).

8.2.3.2 Acclimation to the experimental environment

Table 8-4 Data of $\dot{V}_{O_2}$ heart rate (HR) and VeDBA of incubating king penguins in different environments. Conditions: 1= unstressed in experimental condition, 2= in colony, 3= stressed in experimental condition.

Ind	$\dot{V}_{O_2}$[ml/min]	HR [beats/min]	VeDBA [g]	Conditions
24	55.22	63.12	0.02	1
24	NA	103.88	0.05	2
24	93.34	91.00	0.12	3
25	48.34	87.69	0.04	1
25	NA	74.41	0.12	2
25	68.87	104.00	0.07	3
26	43.91	67.34	0.04	1
26	NA	99.66	0.12	2
26	123.78	129.00	0.20	3
27	49.92	100.56	0.04	1
27	NA	128.54	0.21	2
27	119.28	155.00	0.20	3
28	44.46	38.94	0.07	1
28	NA	64.45	0.09	2
28	75.54	0.29	0.15	3
30	49.74	0.16	0.05	1
30	NA	0.35	0.18	2
30	91.34	0.33	0.17	3

Table 8-5 Data of $\dot{V}_{O_2}$ heart rate (HR) and VeDBA of incubating king penguins at different times after being placed in a respirometer chamber. Time t: time (in hour) when the means of $\dot{V}_{O_2}$, HR and means were made. RMR: 0= $lowest\dot{V}_{O_2}whitin\ 90min$, 1= $lowest\dot{V}_{O_2}$; 3 = $lowest\dot{V}_{O_2}daytime$; 4 = first stable $\dot{V}_{O_2}$ in one hour.

Ind	Start resting	Time t	Stop resting	RMR	$\dot{V}_{O_2}$ [ml/min]	HR [beats/min]	VeDBA [g]
24	18:21:00	18:51:21	08:14:00	0	66.94	63.77	0.03
24	18:21:00	01:48:45	08:14:00	1	54.93	63.42	0.02
24	18:21:00	06:01:51	08:14:00	3	55.22	63.12	0.02
24	18:21:00	19:21:00	08:14:00	4	90.52	86.44	0.04
25	18:55:55	20:01:52	09:37:00	0	48.71	84.86	0.04
25	18:55:55	20:36:07	09:37:00	1	45.94	86.49	0.05
25	18:55:55	07:46:17	09:37:00	3	48.34	87.69	0.04
25	18:55:55	19:55:55	09:37:00	4	56.07	90.92	0.04
26	20:09:00	21:19:28	08:35:00	0	33.73	70.16	0.10
26	20:09:00	21:19:28	08:35:00	1	33.73	70.16	0.11
26	20:09:00	06:13:34	08:35:00	3	43.91	67.34	0.04
26	20:09:00	21:09:00	08:35:00	4	48.31	67.07	0.1
27	18:08:00	19:33:07	08:25:00	0	49.83	109.30	0.05
27	18:08:00	19:33:07	08:25:00	1	49.83	109.3	0.05
27	18:08:00	06:36:33	08:25:00	3	49.92	100.56	0.04
27	18:08:00	19:08:00	08:25:00	4	64.58	113.9	0.12
28	17:52:40	19:06:51	08:21:10	0	42.19	47.96	0.04
28	17:52:40	19:06:51	08:21:10	1	42.19	47.96	0.04
28	17:52:40	07:21:54	08:21:10	3	44.46	38.94	0.07
28	17:52:40	18:52:40	08:21:10	4	48.85	48.84	0.05
30	16:20:08	17:41:41	08:25:26	0	48.24	48.47	0.06
30	16:20:08	05:30:54	08:25:26	1	47.69	40.39	0.17
30	16:20:08	07:05:42	08:25:26	3	49.74	33.92	0.05
30	16:20:08	17:20:08	08:25:26	4	57.51	50.75	0.08

8.2.3.3 Acclimation to the experimental protocol

8.2.3.3.1 Acclimation across walking sessions

Table 8-6 Data of $\dot{V}_{O_2}$ heart rate (HR) and VeDBA of king penguins while walking on a treadmill. Order: 1= first walking session, 2= second walking session, etc.

Ind	Order	$\dot{V}_{O_2}$ [ml/min]	HR [beats/min]	VeDBA [g]
31	1	276.67	140	2.85
31	2	278.84	139	2.96
31	3	295.12	147	3.01
32	1	316.75	125	3.29
32	2	285.55	115	2.98
32	3	280.33	116	3.00
33	1	291.33	150	2.69
33	2	257.47	137	2.50
33	3	248.66	138	2.49
35	1	308.29	163	2.96

35	2	296.16	183	2.74
35	3	281.86	178	2.57
36	1	306.17	168	2.41
36	2	299.60	178	2.46
36	3	308.88	215	2.62
37	1	377.28	152	4.28
37	2	300.10	147	3.13
37	3	266.88	144	2.82

8.2.3.3.2 Acclimation during the first walking session

Table 8-7 Data of $\dot{V}_{O_2}$ heart rate (HR) and VeDBA of king penguins while walking during the first walking session on a treadmill. Min interval: 1= from minute 2 to 5 inclusive, 2= from minutes 6 to 9 inclusive.

Ind	Min interval	$\dot{V}_{O_2}$ [ml/min]	HR [beats/min]	VeDBA [g]
31	1	275.96	147	2.72
31	2	279.49	135	2.95
32	1	330.33	125	3.37
32	2	306.23	127	3.24
33	1	337.92	150	2.54
33	2	256.79	149	2.52
35	1	312.11	162	2.99
35	2	304.11	164	2.93
36	1	329.39	166	2.40
36	2	288.89	169	2.37
37	1	354.73	149	4.81
37	2	369.00	161	4.15

8.2.4 An approach to uncover the cost of pedestrian locomotion: a biomechanical look at the 'optimised fat penguin'

8.2.4.1 Energetics

Table 8-8 Data of $\dot{V}_{O_2}$ and VeDBA of king penguins while walking on a treadmill at different body masses and different speeds.

Ind	Body mass [kg]	Speed [km/h]	$\dot{V}_{O_2}$ [ml/min]	VeDBA [g]
13	11.34	0	85.89	0.09
13	11.34	1	244.72	2.27
13	11.34	1.2	240.36	2.52
13	11.34	1.4	253.42	2.99
13	11.34	1.6	298.24	3.31
14	10.07	0	66.71	0.07
14	10.07	1	183.42	1.88
14	10.07	1.2	197.75	2.19
14	10.07	1.4	209.54	2.58
14	10.07	1.6	218.79	2.93
14	10.56	0	66.82	0.04
14	10.56	1.2	248.03	2.4

14	10.56	1.4	258.99	2.94
14	10.56	1.6	261.43	3.08
14	12.59	0	94.07	0.08
14	12.59	1	257.85	2.28
14	12.59	1.2	266.65	2.6
14	12.59	1.4	279.65	2.98
14	12.59	1.6	308.45	3.42
15	9.67	0	51.78	0.02
15	9.67	1	148.58	1.5
15	9.67	1.2	161.02	1.81
15	9.67	1.4	191.22	2.09
15	9.67	1.6	197.79	2.57
15	10.12	0	58.32	0.02
15	10.12	1	162.98	1.46
15	10.12	1.2	181.15	1.79
15	10.12	1.4	195.97	2.09
15	10.12	1.6	217.74	2.61
15	10.47	1	173.8	1.45
15	10.47	1.2	188.51	1.84
15	10.47	1.4	209.85	2.23
15	10.47	1.6	239.72	2.7
15	12.36	0	71.02	0.02
15	12.36	1	252.04	1.74
15	12.36	1.2	267.93	2.05
15	12.36	1.4	288.37	2.43
16	11.8	0	79.78	0.04
16	11.8	1	221.26	2.15
16	11.8	1.2	223.11	2.45
16	11.8	1.4	239.88	2.81
16	11.8	1.6	268.48	3.26
16	12.61	0	76.7	0.02
16	12.61	1	248.02	2.29
16	12.61	1.2	247.51	2.57
16	12.61	1.4	273.06	2.99
16	12.61	1.6	293.54	3.53
17	10.71	0	67.69	0.03
17	10.71	1	174.85	1.24
17	10.71	1.2	211.04	1.68
17	10.71	1.4	226.33	2.02
17	10.71	1.6	236.55	2.25
17	11.23	1	231.2	1.55
17	11.23	1.2	198.5	1.61
17	11.23	1.4	244.83	2.26
17	11.23	1.6	281.64	2.59
18	11.4	0	77.03	0.27
18	11.4	1	184.13	1.25
18	11.4	1.2	214.14	1.76
18	11.4	1.4	217.46	2.04
18	11.4	1.6	241.83	2.38
18	12.31	0	83.94	0.03

18	12.31	1	207.89	1.52
18	12.31	1.2	201.61	1.9
18	12.31	1.4	239.95	2.47
18	12.31	1.6	275.88	2.69
18	13.56	0	80.63	0.03
18	13.56	1	252.38	2.35
18	13.56	1.2	318.87	3.59
18	13.56	1.4	325.73	3.86
18	13.56	1.6	347	4.33
19	9.17	1	156.52	1.72
19	9.17	1.2	175.14	1.96
19	9.17	1.4	181.74	2.23
19	9.17	1.6	206.46	2.67
19	10.69	0	85.06	0.44
19	10.69	1	218.81	1.96
19	10.69	1.2	223.87	2.27
19	10.69	1.4	263.25	2.78
19	10.69	1.6	260.46	3.14
19	11.56	0	81.62	0.1
19	11.56	1	242.46	2.22
19	11.56	1.2	281.18	2.59
19	11.56	1.4	292.31	2.81
19	11.56	1.6	289.14	3.21
19	12.88	0	108.71	0.06
19	12.88	1	254.42	2.07
19	12.88	1.2	297.48	2.66
19	12.88	1.4	294.34	2.74
19	12.88	1.6	324.87	3.37
20	10.36	0	45.63	0.03
20	10.36	1	139.94	1.54
20	10.36	1.2	161.91	1.86
20	10.36	1.4	168.68	2.28
20	10.36	1.6	180.39	2.75
20	12.05	0	80.1	0.2
20	12.05	1	194.22	1.56
20	12.05	1.2	204.67	2.01
20	12.05	1.4	226.88	2.37
20	12.05	1.6	246.47	2.74
20	14.39	0	108.84	0.03
20	14.39	1	295.91	2.15
20	14.39	1.2	289.06	2.22
20	14.39	1.4	322.4	2.78
20	14.39	1.6	390.11	3.69
21	9.92	0	29.67	0.03
21	11.33	0	64.79	0.02
21	12.23	0	85.14	0.03
21	12.23	1	222.5	1.29
21	12.23	1.2	233.67	1.67
21	12.23	1.6	305.13	2.65
21	13.22	0	100.33	0.08

22	10.99	0	67.84	0.04
22	11.87	0	75.12	0.04
22	11.87	1	215.88	1.98
22	11.87	1.2	271.18	2.7
22	11.87	1.4	253.9	2.65
22	11.87	1.6	262.36	3.01
22	13.15	0	93.29	0.03
22	13.15	1	257.69	1.89
22	13.15	1.2	356.07	2.76
22	13.15	1.4	360.23	3.24
22	13.15	1.6	375.05	3.46

8.2.4.2 Biomechanics

8.2.4.2.1 Video

Table 8-9 Stride and step parameters of king penguins walking on a treadmill at 1.4km/h at different body masses.

Ind	Body mass [kg]	Stand duration [s]	Swing duration [s]	Stride duration [s]	Length [m]	Frequency [s^{-1}]	Step width [m]
19	12.88	0.54	0.25	0.78	0.36	1.28	0.11
19	11.56	0.46	0.23	0.69	0.31	1.46	0.13
19	9.17	0.51	0.26	0.76	0.34	1.33	0.1
20	14.39	0.46	0.23	0.69	0.31	1.46	0.12
20	10.36	0.54	0.26	0.79	0.33	1.26	0.08
21	13.22	0.58	0.28	0.86	0.37	1.18	0.09
21	12.23	0.52	0.26	0.79	0.35	1.28	0.09
21	11.33	0.56	0.28	0.84	0.37	1.19	0.1
21	9.92	0.54	0.28	0.81	0.35	1.24	0.08
22	13.15	0.51	0.26	0.78	0.35	1.3	0.11
22	11.87	0.53	0.28	0.8	0.35	1.25	0.1
22	10.99	0.47	0.24	0.72	0.32	1.4	0.14
22	9.71	0.5	0.26	0.76	0.33	1.33	0.1

8.2.4.2.2 Accelerometry

Table 8-10 Parameters of the Dynamic Body Acceleration (DBA) of king penguins walking on a treadmill at 1.4km/h at different body massed. SD= standard deviation.

Ind	Body mass [kg]	DBA_X		DBA_y		DBA_z			
		Nb peaks/min	amplitude	Nb peaks/min	Amplitude	Nb peaks/min	Amplitude	Frequency [s^{-1}]	frequency SD
13	11.34	161.67	0.63	198.89	0.59	156.11	0.87	1.33	0.16
13	10.68	154.33	0.44	216.67	0.51	153.78	0.55	1.30	0.11
14	12.59	161.67	0.49	209.56	0.60	158.22	0.82	1.35	0.23
14	10.56	161.56	0.45	203.56	0.60	164.89	0.88	1.40	0.18
14	10.07	157.11	0.43	196.11	0.52	153.67	0.84	1.31	0.15
15	12.36	150.56	0.35	195.67	0.67	140.33	0.60	1.21	0.17
15	10.47	144.89	0.46	198.00	0.60	136.78	0.63	1.17	0.10
15	10.12	57.22	0.19	81.00	0.36	56.00	0.34	0.90	0.14
15	9.67	142.81	0.44	196.38	0.56	140.75	0.62	1.22	0.14
16	12.61	161.56	0.50	187.67	0.74	167.33	0.89	1.41	0.13
16	11.8	159.22	0.58	182.78	0.69	165.78	0.76	1.40	0.21
17	12.8	160.11	0.45	162.89	0.68	134.78	0.60	1.22	0.29
17	11.23	137.11	0.38	194.67	0.57	134.67	0.62	1.16	0.16
17	10.71	130.89	0.35	192.78	0.52	135.44	0.55	1.16	0.15
18	13.56	162.00	0.56	194.11	0.81	173.89	1.07	1.48	0.15
18	12.31	145.56	0.41	195.89	0.66	146.33	0.61	1.26	0.24
18	11.4	142.78	0.39	208.56	0.56	142.67	0.60	1.19	0.07
19	12.88	140.89	0.38	198.56	0.64	149.00	0.64	1.32	0.21
19	11.56	154.22	0.45	208.44	0.71	157.56	0.72	1.35	0.20
19	10.69	157.22	0.41	203.22	0.70	156.78	0.71	1.34	0.16
19	9.17	147.78	0.39	185.78	0.60	152.67	0.61	1.30	0.19
21	13.22	144.50	0.36	208.50	0.63	138.88	0.62	1.20	0.09
21	12.23	145.50	0.43	208.70	0.56	145.80	0.75	1.26	0.17
21	11.33	148.33	0.47	209.67	0.58	144.11	0.74	1.21	0.12
21	9.92	151.78	0.51	212.67	0.44	150.67	0.72	1.27	0.15
22	13.15	156.56	0.50	185.44	0.73	147.11	0.90	1.28	0.20

22	11.87	149.00	0.49	215.33	0.66	147.00	0.80	1.26	0.22
22	10.99	169.56	0.69	220.00	0.82	160.89	1.05	1.37	0.15
22	9.71	160.22	0.64	211.33	0.73	150.78	1.13	1.29	0.18
20	14.39	158.33	0.49	203.78	0.66	160.00	0.67	1.38	0.18
20	12.05	150.67	0.43	205.00	0.63	150.44	0.60	1.26	0.10
20	10.36	146.22	0.38	166.67	0.65	148.78	0.64	1.25	0.12

Table 8-11 Parameters of angles (i.e. Static Body Acceleration) of king penguins walking on a treadmill at 1.4km/h at different body masses. SD= standard deviation.

Ind	Body mass [kg]	Roll					Pitch				
		Nb peaks/ min	Frequency [s^{-1}]	SD Frequency	Amplitude	SD Amplitude	Nb peaks/ min	Frequency [s^{-1}]	SD Frequency	Amplitude	SD Amplitude
13	11.34	73.11	0.63	0.10	8.93	2.01	138.89	1.20	0.21	2.90	1.04
13	10.68	75.89	0.64	0.06	8.54	1.29	149.11	1.27	0.15	2.36	0.76
14	12.59	77.11	0.66	0.09	8.34	1.35	137.11	1.19	0.24	2.18	0.78
14	10.56	80.33	0.68	0.10	6.58	1.59	128.67	1.15	0.28	1.89	0.77
14	10.07	73.44	0.63	0.09	8.94	2.16	137.89	1.19	0.21	2.79	1.05
15	12.36	65.44	0.57	0.11	8.69	1.82	97.00	0.87	0.26	2.41	1.00
15	10.47	66.78	0.57	0.06	5.90	0.93	126.67	1.10	0.16	2.37	0.48
15	10.12	27.44	0.26	0.04	2.69	0.52	51.33	0.50	0.09	0.96	0.27
15	9.67	68.06	0.60	0.10	6.91	1.56	131.06	1.15	0.18	2.39	0.78
16	12.61	82.11	0.69	0.08	7.59	1.36	136.44	1.20	0.27	2.16	0.96
16	11.8	81.44	0.69	0.06	6.47	1.01	112.56	1.00	0.27	2.25	0.98
17	12.8	61.33	0.53	0.09	9.21	1.68	98.22	0.88	0.24	2.91	1.18
17	11.23	66.89	0.57	0.06	9.81	1.76	125.11	1.08	0.17	2.75	0.66
17	10.71	66.56	0.56	0.06	9.63	1.78	130.56	1.11	0.15	2.87	0.59
18	13.56	86.44	0.73	0.07	7.73	1.61	137.22	1.21	0.29	2.02	0.97
18	12.31	72.11	0.61	0.06	8.30	1.47	136.33	1.17	0.20	1.80	0.52
18	11.4	71.11	0.60	0.04	7.20	0.93	142.33	1.19	0.07	2.08	0.42

19	12.88	70.00	0.61	0.12	10.52	3.49	121.44	1.09	0.27	2.08	0.90
19	11.56	75.89	0.65	0.09	10.19	2.02	105.00	0.95	0.29	2.37	0.97
19	10.69	76.44	0.65	0.09	11.53	2.32	137.11	1.20	0.25	2.13	0.62
19	9.17	74.67	0.63	0.07	8.65	2.17	146.56	1.25	0.18	2.17	0.79
21	13.22	64.88	0.57	0.07	7.51	0.76	136.63	1.18	0.10	1.47	0.33
21	12.23	72.00	0.62	0.06	10.94	4.67	133.00	1.16	0.17	2.13	1.03
21	11.33	71.22	0.60	0.05	3.63	0.46	142.11	1.20	0.12	0.94	0.28
21	9.92	74.33	0.63	0.05	8.19	1.36	143.67	1.22	0.14	2.17	0.80
22	13.15	71.00	0.61	0.10	12.51	2.54	118.00	1.05	0.26	2.95	1.81
22	11.87	68.56	0.59	0.08	9.80	1.50	133.67	1.14	0.18	2.46	0.94
22	10.99	78.00	0.66	0.07	8.68	1.41	113.44	1.02	0.27	2.84	1.40
22	9.71	74.11	0.63	0.07	12.59	2.12	102.22	0.92	0.27	4.96	2.30
20	14.39	79.00	0.67	0.08	7.69	1.38	138.89	1.22	0.25	1.70	0.75
20	12.05	74.33	0.62	0.04	7.15	0.72	150.22	1.25	0.09	1.73	0.38
20	10.36	73.33	0.62	0.04	8.44	0.69	148.44	1.24	0.07	2.29	0.55

8.2.5 It costs to be late: investigating the onshore energy expenditure of incubating king penguins.

Table 8-12 Data of $\dot{V}_{O_2}$ king penguins while walking on a treadmill at different body masses, different speeds and inclines.

Ind	Body mass [kg]	Speed [km/h]	Incline	$\dot{V}_{O_2}$ [ml/min]
14	10.07	1	1	183.42
14	10.07	1.2	1	197.75
14	10.07	1.4	1	209.54
14	10.07	1.6	1	218.79
14	10.07	1	2	208.30
14	10.07	1.2	2	225.66
14	10.07	1.4	2	246.12
14	10.07	1.6	2	258.49
14	12.59	1	1	257.85
14	12.59	1.2	1	266.65
14	12.59	1.4	1	279.65
14	12.59	1.6	1	308.45
14	12.59	1	2	286.71
14	12.59	1.2	2	294.51
14	12.59	1.4	2	328.53
14	12.59	1.6	2	345.13
15	9.67	1	1	148.58
15	9.67	1.2	1	161.02
15	9.67	1.4	1	191.22
15	9.67	1.6	1	197.79
15	9.67	1	2	175.59
15	9.67	1.2	2	196.77
15	9.67	1.4	2	216.47
15	9.67	1.6	2	243.12
15	12.36	1	1	252.04
15	12.36	1.2	1	267.93
15	12.36	1.4	1	288.37
15	12.36	1	2	286.70
15	12.36	1.2	2	296.12
15	12.36	1.4	2	355.90
18	13.56	1	1	252.38
18	13.56	1.2	1	318.87
18	13.56	1.4	1	325.73
18	13.56	1.6	1	347.00
19	9.17	1	1	156.52
19	9.17	1.2	1	175.14
19	9.17	1.4	1	181.74
19	9.17	1.6	1	206.46
19	9.17	1	2	185.11
19	9.17	1.2	2	198.53
19	9.17	1.4	2	204.26
19	9.17	1.6	2	226.22
19	12.88	1	1	254.42

19	12.88	1.2	1	297.48
19	12.88	1.4	1	294.34
19	12.88	1.6	1	324.87
20	10.36	1	1	139.94
20	10.36	1.2	1	161.91
20	10.36	1.4	1	168.68
20	10.36	1.6	1	180.39
20	10.36	1	2	189.90
20	10.36	1.2	2	186.54
20	10.36	1.4	2	210.61
20	10.36	1.6	2	240.39
20	14.39	1	1	295.91
20	14.39	1.2	1	289.06
20	14.39	1.4	1	322.40
20	14.39	1.6	1	390.11
20	14.39	1	2	307.20
20	14.39	1.2	2	344.63
20	14.39	1.4	2	393.36
20	14.39	1.6	2	419.08
21	9.92	1	2	195.94
21	9.92	1.2	2	180.37
21	9.92	1.4	2	205.50
21	9.92	1.6	2	239.62
21	13.22	1	2	295.51
21	13.22	1.2	2	339.02
21	13.22	1.4	2	339.96
21	13.22	1.6	2	392.68
22	13.15	1	1	257.69
22	13.15	1.2	1	356.07
22	13.15	1.4	1	360.23
22	13.15	1.6	1	375.05
22	13.15	1	2	265.17
22	13.15	1.2	2	301.11
22	13.15	1.4	2	342.21
22	13.15	1.6	2	365.41

Table 8-13 Distance and cost of the likely shortest and longest routes into the colony. Distance and cost are split into flat, incline and stressed (when crossing breeding areas). Colonies: A= Ile aux Cochons (Crozet Archipelago), B= Ratmanoff (on the Kerguelen Archipelago), C= Jardin Japonais and D= La Baie du Marin (both on Possession Island, Crozet Archipelago). J = journey from sea to zone of attachment, R= return journey from zone of attachment to sea.

Colony	type	outward	Distance [m]						Cost [LO_2]							
			flat		incline		Stressed		flat		Incline		Stressed		Total	
			J	R	J	R	J	R	J	R	J	R	J	R	J	R
A	Short	1	0		1		131		0.0		0.0		2.0		2.0	
A	Short	2	0	0	8	10	124	255	0.0	0.0	0.1	0.1	1.9	3.8	2.0	4.0
A	Long	1	1305		1044		256		19.0		17.8		3.9		40.7	
A	Long	2	1809	3114	540	1585	256	511	15.6	34.7	5.5	23.3	3.9	7.7	25.0	65.7
B	Short	1	0		0		155		0.0		0.0		2.3		2.3	
B	Short	2	0	0	0	0	155	311	0.0	0.0	0.0	0.0	2.3	4.7	2.3	4.7
C	Short	1	0		41		0		0.0		0.7		0.0		0.7	
C	Short	2	0	0	0	41	41	41	0.0	0.0	0.0	0.7	0.6	0.6	0.6	1.3
C	Long	1	189		141		152		2.8		2.4		2.3		7.5	
C	Long	2	308	497	23	164	152	305	2.7	5.4	0.2	2.6	2.3	4.6	5.2	12.7
D	Short	1	0		0		30		0.0		0.0		0.4		0.4	
D	Short	2	0	0	6	6	23	53	0.0	0.0	0.1	0.1	0.4	0.8	0.4	0.9
D	Long	1	140		156		129		2.0		2.6		1.9		6.6	
D	Long	2	241	380	54	210	129	258	2.1	4.1	0.6	3.2	1.9	3.9	4.6	11.2

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